The Genetic Epidemiology of Age-related Hearing Impairment

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The Genetic Epidemiology of Age-related Hearing Impairment

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Doctor of Philosophy in Genetics & Molecular Medicine Research

To my parents

for always believing in me.

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Abbreviation	
ARHI	age-related hearing impairment
K^+	sodium
Ca^{++}	calcium
dB HL	decibel hearing level
PTA	pure-tone average
Hz	hertz
pta	pure-tone audiogram
PTT	pure-tone threshold
OR	odds ratio
SNHL	sensorineural hearing loss
Z_{high}	Z-score calculated for the high frequencies
95%CI	95% confidence interval
MZ	monozygotic
DZ	dizygotic
ICCs	intra-class correlations
NAT2	N-acetyltransferase 2
LD	linkage disequilibrium
SNP	single nucleotide polymorphism
LOD	logarithm of the odds
PCA	principal component analysis
PC	principal component
GWAS	Genome-wide association study
ROS	reactive oxygen species
WHO	world health organization
HL	hearing loss
BEHL	better ear hearing threshold level
SEM	structural equation modelling
PCR	probandwise concordance rate

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Abstract

Age-related hearing impairment (ARHI) affects 46% of the population over the age of 48 and with increasing life expectancy in Western nations, this incidence is likely to rise. The causes for this disorder are still poorly understood but there is known to be a heritable component of around 65%. In addition, epigenetic regulation of gene expression changes over time and may explain many age-related traits. The basis of this research was to explore genetic and epigenetic factors in ARHI to understand better the mechanisms involved in its pathology.

Hearing data were obtained from female volunteers of the TwinsUK register using the gold standard measure, air-conduction pure-tone audiometry and a web-based speech-in-noise perception test. The prevalence of ARHI in TwinsUK was comparable to previous reports. Heritability estimates based on the classical twin model confirmed a moderate heritability of hearing ability in TwinsUK, supporting the use of this sample in genetic association studies. Genome-wide association with hearing ability was performed in TwinsUK but no genome-wide significant polymorphisms were identified possibly due to inadequate sample size. Accordingly, the data were combined with existing genome-wide association studies (GWAS) of hearing function from the G-EAR consortium. This meta-analysis resulted in a genome-wide significant association with an exonic SNP of the *SIK3* gene encoding salt-inducible kinase 3, a novel gene reported to regulate metabolism and skeletal development via *HDAC4*. Immunohistochemistry of *sik3* in mouse models confirmed striking expression profiles in hair cells and spiral ganglion cells of mouse cochlea, validating a putative function of *SIK3* in hearing ability.

A small epigenome-wide association study of hearing ability in TwinsUK (n=115) revealed an epigenome-wide significant association with a probe in the promoter region of *TCF25*. Epigenome-wide associations at two highly associated probes (*TCF25* and *POLE*) were replicated in an independent sample from TwinsUK (n=203). DNA methylation at these genes was negatively correlated with expression of the same, indicating gene expression repression by DNA methylation. Furthermore, using identical twins discordant for hearing loss, differentially methylated regions were found at genes *ACP6* and *CCNDBP1*.

This research supports a role of common genetic variants in ARHI, including the novel association with *SIK3,* which may be essential for healthy hair cell development and maintenance of spiral ganglion cells with increased age. Differentially methylated

regions were significantly associated with ARHI and showed an effect on gene expression despite small sample size, supporting a role of epigenetic modifications in ARHI. This research is the first to report genome-wide significant association with *SIK3* and epigenome-wide significant associations at *TCF25* with ARHI, which may shed light on the pathways involved in this disabling condition.

Chapter 1: Introduction

Abstract

In 2009, 16% of all British citizens were aged 65 years or older. This number is expected to rise up to 23% by 2034 [1], highlighting the impact of ageing on the population. Higher life expectancy will inevitably be accompanied by an increased incidence of age-related disorders, some of which are still rarely studied. Better understanding of age-related traits, like age-related hearing impairment (ARHI), is essential to enable treatment or possibly prevention of these disorders. Loss of hearing ability can affect simplest everyday activities, such as day to day conversations with other people, leading to social isolation [2], loss of physical and mental wellbeing and ultimately incapability to work. In the March 2006, 9 million individuals in the UK were estimated to be deaf or hard of hearing. Most of these subjects experienced hearing loss (HL) as a part of the ageing process [3].

This chapter introduces the anatomy of the ear and known pathologies of ARHI. In addition a review of the current literature covering environmental risk factors for ARHI as well as the basic rational of human ageing and studies on the latter as well as the role of twins in medical research are discussed.

Anatomy and physiology of the ear and hearing

Anatomy of the ear

The ear can be divided into three major parts, the outer, middle and inner ear (Figure 1)[4]. Main structures of the outer ear include the pinna and ear canal. The pinna represents the visible part of the ear extruding from both sides of the head. This structure leads in to the ear canal, embedded in the mastoid bone. This narrow passageway leading to the eardrum extends on average to 2.4 cm [5]. While the inner two thirds of the ear canal have a bony wall, the outer third has a wall formed of cartilage lined by skin tissue including a high proportion of hair and ceruminous glands [5]. An opaque membrane called the tympanic membrane or eardrum made of squamous stratified epithelium, a fibrous middle layer and a mucosa lining the middle ear cavity defines the border between outer and middle ear.

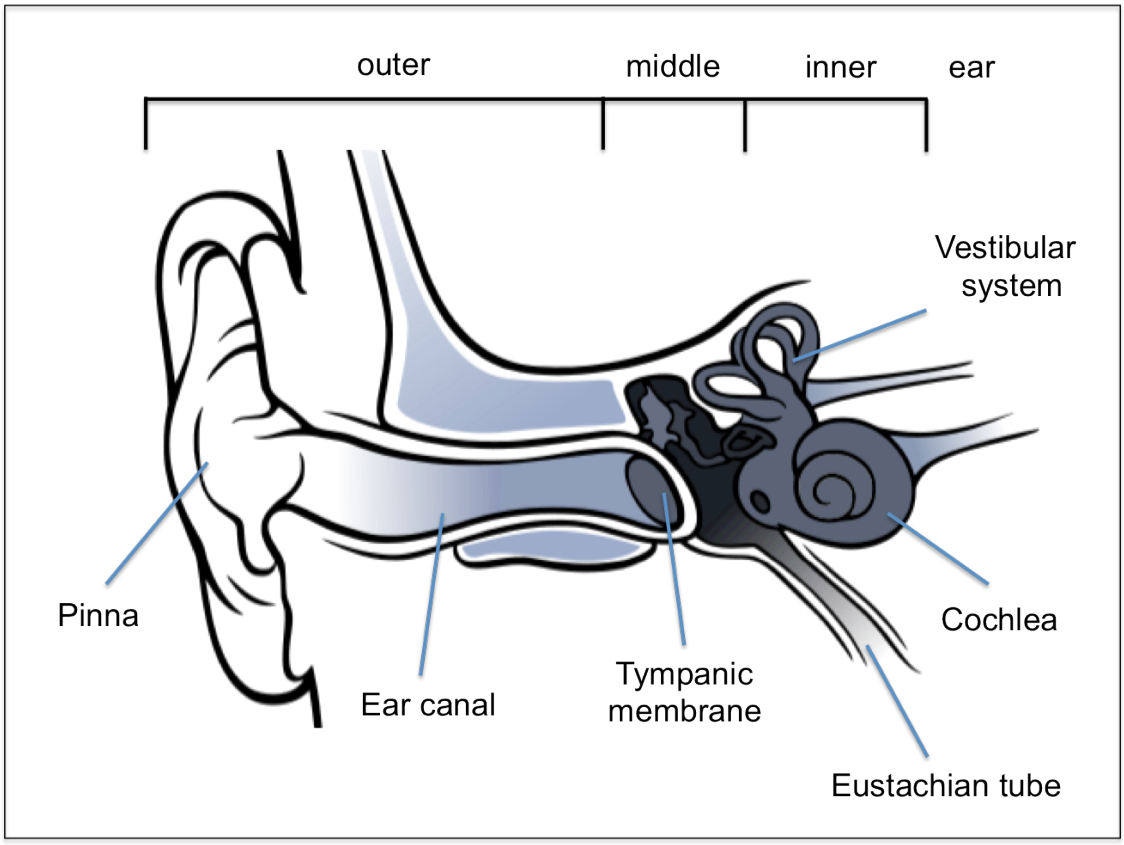


Figure 1 Anatomy of the human ear

Schematic drawing of the major components of the human ear. For descriptive purposes, the ear is divided into outer, middle and inner ear.

Behind the tympanic membrane an air filled cavity, the middle ear is located, which contains the three auditory bones (malleus, incus and stapes). Like the name implies, the middle ear forms the connection between outer and inner ear, with signals being transmitted via the ossicular chain of auditory bones. These three tiny bones form a tight but flexible link, with membranes and ligaments supporting their function. While the malleus connects to the tympanic membrane, stapes touches the wall of the inner ear at the oval window, one of two membrane-lined windows linking the middle ear cavity to snail shaped structure of the inner ear. The flexible connection between the ossicular bones is essential for transmission of vibration from the tympanic membrane to the oval window and is further regulated by two muscles, the tensor tympani and the stapedius muscle. Ventilation of the air-filled middle ear cavity is provided via the Eustachian tube, a canal opening the middle ear to the sinuses. Despite enabling ventilation this passage also presents a way to clear the inner ear from debris and an entry for bacteria to the middle ear, the main cause of otitis media [6]. Further structures of the middle ear include the facial and chorda tympani nerve.

The inner ear is embedded in the temporal bone and formed by a connection of fluid filled cavities and channels. Normally, the inner ear is functionally divided into cochlea

and semicircular channels, with the cochlea being responsible for sound processing and the semicircular channels controlling balance. This short summary of the anatomy of the inner ear will be focussed on the cochlea. The structure of the cochlea resembles that of a tapered spiral, giving its name, which translates as snail or screw. On the inside, three channels run in parallel from the base to the apex, the scala vestibuli, scala media and scala tympani (Figure 2). Scala vestibuli and scala tympani are filled with perilymph (high in sodium), while the scala media or cochlear duct, located between the two other chambers, contains endolymph (high in potassium)[7].

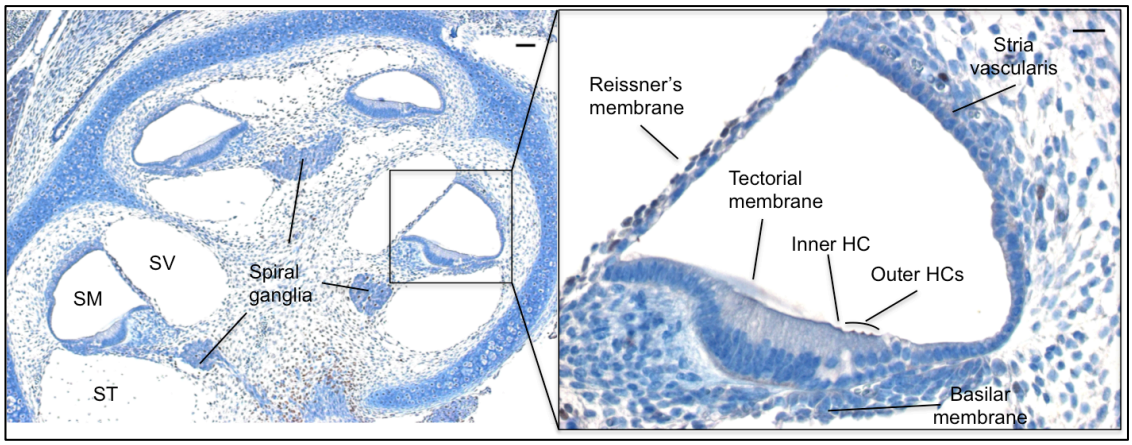


Figure 2 Cochlea and the organ of Corti

On the left hand side a light microscopic cross-section through the mouse cochlea at the day of birth is shown (SV= scala vestibuli; SM=scala media; ST=scala tympani). The scala media containing the organ of Corti is depicted at a higher magnification on the right hand side (HC=hair cell).

The cochlear duct is outlined by three structures, forming a triangular shape; the basilar membrane, the stria vascularis and Reissner's membrane (Figure 2). Based on the basilar membrane lies the organ of Corti, which harbours the sensory inner and outer hair cells, converting a mechanical signal from the sound waves travelling through the fluid of the cochlea to an electric one, giving a neuronal stimulus to the auditory nerve. The basal part of the hair cells is connected to the basilar membrane whereas the apical part is bathed in endolymph with occasional contact to the tectorial membrane. The organ of Corti contains three rows of outer hair cells but only one row of inner hair cells. While inner hair cells transmit the primary sound signal, outer hair cells are important for amplification of the same [8,9]. The hair cells derived their name from the arrangements of stereocilia on the apex of these cells, facing the endolymph.

The stereocilia are arranged in a staircase like fashion giving the impression of a "V" when viewed from the top of the hair cell [10]. Links between adjacent stereocilia are responsible for maintenance of this structure and their role in opening in ion channels

has been suggested. Different stereocilia links have been reviewed in [11]. Disturbance of this strict arrangement and progressive degradation of stereocilia has been detected in several mouse models for HL [12,13]. Stereocilia are actin based structures and therefore relatively stiff [14]. To allow more flexibility, the basal ends of the stereocilia are anchored at the cuticular plate on top of the hair cell via a flexible taper [15]. Several unconventional myosins (myosin 15a [16],myosin 3a [17] and myosin 7a [18]) have been implicated in stereocilia growth and lack or mutation of the same is a common cause of HL [17,19,20]. Deflection of the stereocilia leads to opening or closing (depending on the direction of deflection) of transduction channels, a process referred to as mechanotransduction [11].

Auditory nerves form the connection between the basal end of the hair cells and the spiral ganglion. Two types of afferent (leading to the brain) spiral ganglion neurons are known, differing in anatomy and cells they innervate [21]. Type 1 spiral ganglion neurons innervate solely single inner hair cells and show a thick radial appearance, whereas type 2 neurons are thinner and connect to multiple outer hair cells [21,22]. Both inner and outer hair cells are further connected to efferent (coming from the brain) nerve fibres, providing feedback to the sensory cells. Afferent cochlear nerves transmit signals to the cochlear nucleus located in the brainstem, from where it is sent via the superior olivary nucleus, inferior colliculus and medial geniculate nucleus to the primary auditory cortex located in the temporal lobe.

Other essential features of the cochlear duct include the stria vascularis, a membrane rich in ion channels, which ensure the correct chemical composition of the endolymph corresponding to a positive endocochlear potential of +80mV [23,24]. The stria vascularis can be divided into three layers: marginal, intermediate and basal cells. Ion channels of the stria vascularis are located in the marginal part of the stria, facing the endolymph, whereas the intermediate layer of the stria contains capillaries to provide perfusion of these ion channels. The intermediate and basal layers form a tight seal of the scala media by the use of gap junctions (i.e. connexins)[25].

Physiology of the ear and hearing

Sound waves entering the ear at the pinna are led via the ear canal towards the tympanic membrane. The pinna can help to localise sound, however this effect is limited in humans compared to other animals, like cats or dogs, which display larger and more motile pinna. The specific resonance property of the ear canal results in enrichment of the sound signal at frequencies ranging from 1500Hz to 6000Hz [5].

Sound waves reaching the tympanic membrane are transformed into vibrations of the latter causing a movement of the ossicular chain, directly attached to the tympanic membrane. This movement going from malleus to incus and stapes exerts pressure on the oval window due to stapes footplate pushing on it. The oval window is a membrane lining the basal part of the cochlear canals. Stretching of the oval window creates a wave in the fluid perilymph, which travels along the snail-shaped cochlea. While travelling through the cochlea the wave builds up until it reaches its maximum. The location, where the sound wave reaches its maximum is defined by its initial pitch. This explains the tonotopical organisation of the cochlea; while low-pitched sounds reach the maximum at the apex of the cochlea, high pitched sounds peak at its base. At this point, the wave travelling underneath the basilar membrane will cause a maximum distortion of the membrane, pushing the membrane and hair cells residing on it upwards against the tectorial membrane. Stereocilia located on the apical surface of the hair cells form the point of contact with the tectorial membrane. As the hair cells are pushed upwards, stereocilia are deflected towards the stria vascularis, triggering the opening of mechanical ion channels on top of the stereocilia to open. K^+ ions from the endolymph enter the stereocilia upon channel opening causing depolarization of the hair cell. Depolarization of the hair cell results in opening of voltage-gated ion channels at the hair cell membrane causing K^+ efflux and Ca^{++} ions entering the cell. Ca^{++} ions in the hair cell trigger the discharge of glutamate, the main excitatory neurotransmitter in the inner ear. Negative deflection of the stereocilia can lead to closing of ion channels. While inner hair cells innervate afferent neurons via glutamate release, outer hair cells are mainly innervated by efferent neurons. Outer hair cells fulfil fine-tuning mechanisms by changing their length and shape [26]. Depolarization and motility of outer hair cells results in movement of the basilar membrane [26]. Changes in shape of outer hair cells are dependent on prestin [27,28], motor proteins and acetylcholine [29]. The quantity of neurotransmitter released by inner hair cells is directly proportional to the depolarization and Ca^{++} influx.

Hearing Loss and ARHI

Forms of hearing loss

HL is a very broad medical field that is defined by its' heterogeneity in forms of manifestation, causal factors and the corresponding diagnostic tests. This paragraph aims to give a short overview of the variety of HL and map ARHI in this field.

Forms and manifestations of HL can be divided by the age of onset (congenital, pre-lingual, before adulthood, adult-onset and age-related) and whether the HL occurs in combination with other pathologies (syndromic HL) or on its own (non-syndromic). Knowledge about the affected structures in the ear allows further division into conductive, sensory or neural HL. Conductive HL is caused by a dysfunction or impairment of the outer and middle ear. In this form of HL, the conduction of sound to the cochlea is impaired. Causes of conductive HL include Otosclerosis and Cholesteatoma or other damage to the outer and middle ear. Sensory and neural HL are often summarised as one (sensorineural HL), and refer to damage to structures of the cochlea and auditory nerve, respectively. Most definitions of sensory HL refer to damage or loss of sensory hair cells in the cochlea, however also damage to other structures (i.e. stria vascularis) of the cochlea can lead to this impairment.

HL can be caused by multiple factors, which can broadly be divided into genetic predisposition and acquired environmental exposure, or a combination of both. Environmental risk factors include, among others, noise exposure, ototoxic medication and infections affecting the ear, and have been determined in various epidemiological studies of HL, which will be covered in more detail below. However, infections of the ear might also be influenced by genetic predisposition [30].

According to the hereditary HL homepage [31] about 100 genetic loci have been associated with HL. However, many of these loci are very rare and only account for HL in one or a few families. This suggests a high genetic heterogeneity for the phenotype. Genetic loci for HL are named according to their mode of inheritance: autosomal dominant (DFNA), autosomal recessive (DFNB) or X-linked (DFN); followed by a number corresponding to the order of detection [32] . The candidate genes identified from these loci fulfil a variety of functions in the inner ear, including unconventional myosins, cytoskeletal proteins, transcription factors, gap junction and extracellular matrix proteins [31]. Nevertheless, the function of several of the associated HL genes and loci is still unknown. A regularly updated list of HL loci with links to the corresponding literature can be found at the hereditary HL website [31].

Hearing ability can be assessed via various hearing tests. The current standard hearing test is the pure-tone audiogram (pta). This test is often referred to as a “subjective” test, as it relies on a behavioural response of the test subject. The pta determines the lowest sound intensity (measured in decibel HL (dB HL)) an individual can hear for different frequencies (measured in hertz (Hz)) (Figure 3) and will be covered in more detail in the following chapters. The sound intensities measured for each ear and frequency are

referred to as pure-tone thresholds (PTTs). Hearing impairment is measured in decibel HL, which corresponds to the decibels an individual's hearing ability diverges from healthy hearing. The world health organization (WHO) grades hearing impairment into mild, moderate, severe and profound HL depending on the range of HL. Grades of hearing impairment adapted from the WHO and corresponding hearing performances are listed in

Table 1.

Table 1 Grades of hearing impairment

grade of hearing impairment	audiometric ISO value for the better ear [dB HL]	hearing performance
no impairment	≤25	Healthy hearing ability. Able to hear whispers.
mild	26-40	Able to hear and repeat words spoken in normal voice at 1 metre.
moderate	41-60	Able to hear and repeat words spoken in raised voice at 1 metre
severe	61-80	Able to hear some words when shouted into better ear.
profound	>80	Unable to hear and understand even a shouted voice

Grades of hearing impairment as adapted from the WHO [33] are listed by corresponding range of pure-tone threshold that can be perceived in the better hearing ear and expected hearing performance.

Newest data from Action on Hearing Loss [34] suggests that one sixth of the UK population (~10,130,000 individuals) is affected by HL, of which 800,000 are profoundly hearing impaired or deaf [35]. 41.7% of all adults aged 50 years or older suffer from mild (>25 dB HL) to profound (>80 dB HL) hearing impairment, highlighting the prevalence of ARHI.

Phenotypes and Pathologies of ARHI

ARHI describes the progressing HL acquired with age. Data from an Italian cohort has mapped the average age of onset to 40 years [36]. Figure 3 and Figure 4 show example air-conduction ptas of a healthy hearing subject and an older adult affected by ARHI, respectively. In a healthy hearing individual, PTTs are low (<40 dB) over all frequencies. In most ARHI cases, HL progresses from the higher to the lower frequencies, causing a characteristic down-slope in the pta (Figure 4). However, other characteristic forms of ARHI have been described, causing primary HL in other frequency ranges than the higher frequencies [37]. Both ears are expected to be affected equally by this disorder. Men are more likely to be affected by age-related HL than women (possibly due to increased exposure to occupational noise or differences

in hormone signalling [38,39]), but it needs to be considered that women have a higher life expectancy than men (78 years for men, 82 years for women, UK, 2009 [40]) , thereby raising the prevalence of female subjects with ARHI in the oldest age groups.

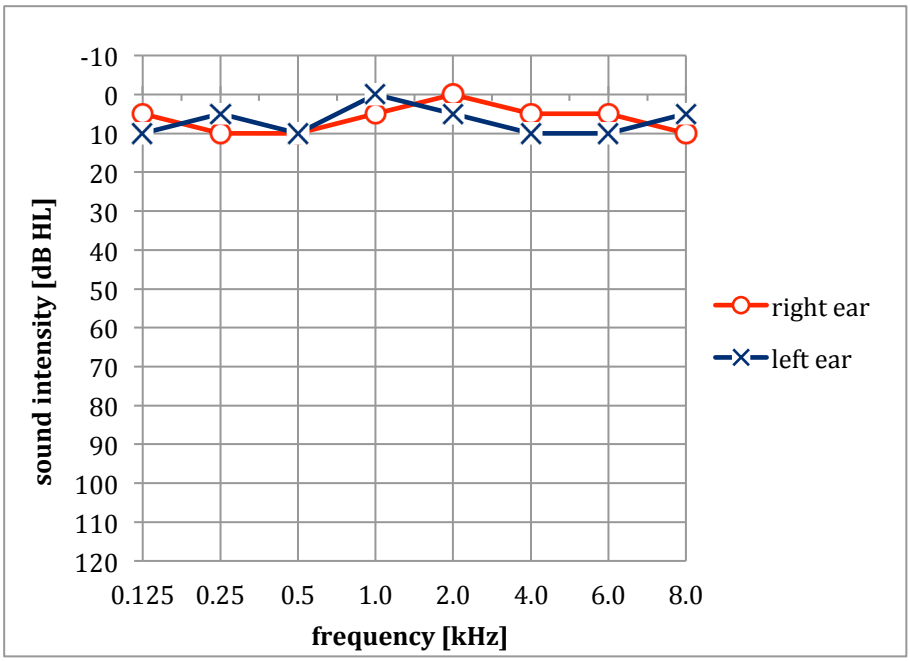


Figure 3 Example air-conduction pure-tone audiogram of a healthy hearing individual

The pure-tone audiogram measures the lowest sound intensity an individual can hear at different frequencies. Measurement is performed separately for both ears. Pure-tone thresholds for the right ear are depicted as red circles and pure-tone thresholds of the left ear are shown as blue crosses. The audiogram of a healthy hearing individual shows low sound intensities (≤ 25 dB HL) over all frequencies.

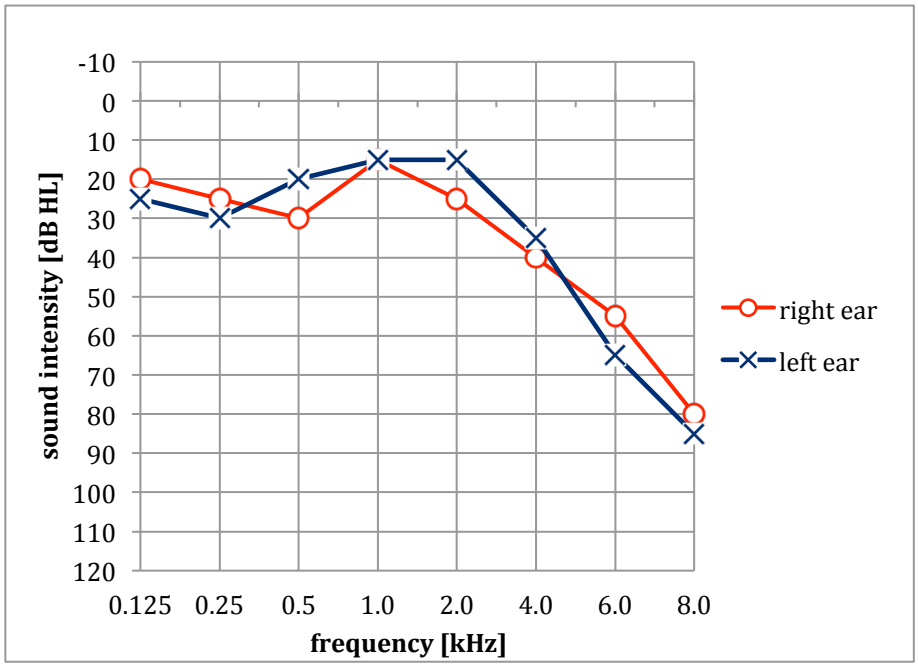


Figure 4 Example air-conduction pure-tone audiogram of an individual affected by ARHI

The pure-tone audiogram measures the lowest sound intensity an individual can hear at different frequencies. Measurement is performed separately for both ears. Pure-tone thresholds for the right ear are depicted as red circles and pure-tone thresholds of the left ear are shown a blue crosses. The audiogram of an individual affected by ARHI shows a characteristic down-slope in sound intensities for the higher frequencies.

Reported changes to the ageing inner ear and the corresponding pathology of ARHI is primarily based on the histological light microscopy observations conducted on the cochlea of 17 individuals with various types of age-related HL [41,42]. Though these observations could not really be reported as recent, they are still the most cited ones in regards to ARHI pathology.

Schuknecht et al described four different pathological sub-phenotypes: sensory, neural, metabolic or strial and mechanical ARHI. Sensory ARHI is the most prevalent type accounting for 50% of all cases. It is defined by a loss of sensory hair cells particularly in the basal part of the cochlea. The loss of hair cells was associated with damage to supportive cells and spiral ganglion neurons, however, the loss of neurons was assumed to be a secondary effect to loss of supportive and hair cells. Sensory hair cells in the basal part of the cochlea respond to sound stimuli of higher frequency, whereas the lower frequencies innervate hair cells in the apical part [43,44]. Individuals with above described pathology showed HL for the higher frequencies, which corresponded to hair cell loss in the basal part of the cochlea.

Strial ARHI, also referred to as metabolic, is characterized by an atrophy of the stria vascularis, causing a flat slightly descending audiogram. The stria vascularis is the metabolic centre of the cochlea and contains a high density of ion-channels, responsible for the correct ion concentration of the endolymph. Atrophy of this tissue would lead to an imbalance of ion concentration and therefore influence the high electrical potential inside the scala media. This imbalance would affect all areas of the cochlea, thus resulting in HL over all frequencies (flat audiogram). This theory is supported by the flat audiogram seen in patients with endolymphatic hydrops and Meniere's disease [45].

Neural ARHI is associated with loss of neurons both in the spiral ganglion and auditory pathways [42]. The loss of neurons is assumed to start early in life without any phenotypic symptoms. Hearing ability is only affected after a threshold of neuronal loss is exceeded. In most cases, the sensory hair cells in the cochlea remain unaffected of these changes. Individuals with this pathology often show normal pure-tone audiograms (due to the healthy hair cells), but report difficulties in word discrimination, indicating problems in sound signal processing.

The pathology of mechanical or cochlear conductive ARHI is not defined. *Schuknecht et al* suggested a stiffness of the basilar membrane leading to this pathology; however, light microscopy was not sufficient to prove this hypothesis. Individuals with this

hypothetical pathology show a slightly descending pure-tone audiogram. It was further highlighted that most affected individuals show a mixture of the described pathologies, addressed as mixed ARHI [41].Newer data from the observations of 21 temporal bones of individuals with ARHI revealed that loss of hair cells (inner and outer), loss of stria vascularis volume and reduction in spiral ganglion cells were significantly associated with a down-sloping audiogram, supporting a mixed form of ARHI [46].

Current treatment strategies and technologies for ARHI

Few treatment strategies for ARHI are available but due to their relative improvement to hearing ability and therefore quality of life in higher age, they are worth mentioning [47]. The most common used appliance is a hearing aid. Hearing aids take up sound waves and convert them into electric stimuli. Modern hearing aids can be personalised to the specific needs of the patient. In case of high frequency HL, sounds in the affected frequencies can be shifted to lower frequencies or be amplified particularly. In addition, hearing aids can be set to reduce background noise and adapt automatically to certain situations/ environments. However, the choice of hearing aid should also consider the users cognitive and fine motor abilities to handle their new device. Despite major benefits to hearing ability reported by most hearing aid users and described in various publications [48,49], many elderly refrain from using hearing aids due to cosmetic reasons. Wearing a hearing aid is unfortunately still associated with old age instead of regain of hearing ability and associated freedom. In addition, fitting and acclimatisation to of a new hearing aid can be tedious and first disappointments might result in rejection of the hearing aid. In general, hearing aids should be used rather earlier than later, as the change from deafness to sudden hearing ability regain can be very harsh.

Cochlear implants offer a different mechanism to restore hearing ability. In this case electric stimuli are formed from sound waves and directly used to excite the auditory nerve. However, this method is more intrusive, as it requires surgical intervention, and a healthy auditory nerve for success. The cochlear implant is fitted close to the head behind the pinna and can often be covered by hair, making it more favourable cosmetically. Good results for ARHI affected individuals have been reported with cochlear implants [50,51]. However, this treatment is still relatively rarely used in ARHI patients due to the associated medical risks and high treatment costs.

Further options include hearing assistive devices like telecoils which can be combined with hearing aids or cochlear implants. Telecoils are tiny coils of wire fitted into specific

hearing aids, which can pick up magnetic signals set up for it. Telecoils are often used in supermarkets or other public places, in addition, special phones with telecoil option have been developed. To use the telecoil, the setting of the hearing aid has to be changed from microphone to telecoil with might be difficult for older users. Other hearing assistants can alert the user of missed sounds by visual, tactile or audiologic triggers. Further assistive devices amplify the sound similar to a hearing aid.

The successful application of a hearing aid or cochlear implant is dependent on 4 factors: competent fitting of the device, motivation and willingness of the patient to use and adapt to the new technology as well as the ability of the patient to understand and handle its new aid. Consideration of these factors can give remarkable results in hearing improvement; although it does it will not fully restore healthy hearing ability.

Currently there are no pharmaceutical treatments available for age related hearing impairment. However, future drugs could be targeted at age-related changes in the cochlea and the audiologic neurons connected to it. One possibility would be to restore the expression of inhibitory neurotransmitters, whose expression has been shown to be reduced in aging rats [52]. Counteracting this reduction would help to preserve the balance of excitation and inhibition of audiologic neurons. Furthermore, overproduction of excitatory neurotransmitter was shown to have a ototoxic effect on synapses between hair cells and neurons of the spiral ganglion [53].

Epidemiological population-based studies on ARHI

Several epidemiological population-based cohorts have been collected in the last decades to determine environmental and genetic risk factors for ARHI. This paragraph is meant to give a short overview of the major cohorts available and the data collected for the various studies. Specific information on each cohort is summarised in Table 2.

The Framingham Heart Study was started in 1948 as a longitudinal population based cohort of the citizens living in Framingham, Massachusetts. This study was created to study the epidemiology of cardiovascular diseases in previously healthy individuals on a biennial basis [54]. Tests for ARHI were performed at examination 18 of the cohort. The tests included pure-tone audiometry as well as word recognition tests and self-reported HL. Besides investigating environmental risk factors [55], heritability [38] and linkage studies [56] of ARHI have been performed for subjects from the Framingham cohort.

The Epidemiology of HL study was initially founded as a study focussing on ophthalmologic traits. Subjects for this cross sectional study were recruited from Beaver Dam, Wisconsin [57]. The cohort was extended to an offspring cohort, focussing on the children of the original participants [58]. Both cohorts covered pure-tone audiometry (air- and bone-conduction) and self-reported HL. The main focus of these two related cohorts was to identify the prevalence of ARHI and determine non-genetic risk factors, justifying the high number of environmental measures recorded [2,57,58,59,60,61]. It should be noted that the Beaver Dam offspring cohort is relatively young in respect to the normal age for cohorts on age-related traits.

The Blue Mountains Hearing Study is based on a 5-year follow up of the Blue Mountains Eye Study cohort. Participants for this cohort were recruited from west of Sydney, Australia [62]. This study covers pure-tone audiometry; self-reported HL and the Hearing Handicap Inventory for the Elderly-Screening Version (HHIE-S) test. Bone conduction audiometry was only conducted in selected participants. This cohort focuses on both environmental risk factors and the sensitivity of self-reported HL and the HHIE-S in comparison to pure-tone audiometry [62,63,64].

The ARHI study is a multicentre-study based on data collected in eight European Ear-Nose and Throat departments. This cohort was established to study environmental, medical and genetic risk factors of ARHI. Publications are available concerning various environmental risk factors [65,66], candidate gene [67,68], linkage studies [69,70] and genome-wide association studies [70,71]. The phenotypes used to summarize HL vary from pure-tone averages for different frequency ranges and standardised Z-Scores [72] to principal components of hearing ability (PC1; PC2; PC3). These different hearing measures will be referred to in more detail in chapter 2.

The Finnish Twin study on ageing was created as a study on older twins from the Finnish twin registry [73]. The Finnish twin study on ARHI, a part of the general ageing study, included exclusively female twin pairs. Hearing ability was summarized as a better ear hearing threshold (BEHL) calculated from pure-tone air conduction thresholds. In addition, hearing data was obtained in form of a speech discrimination test as well as self-reported HL [74,75].

It should be noted that hearing data collected for the Epidemiology of Hearing Loss studies (Beaver Dam and Beaver Dam offspring cohort) use hearing measures for the worse ear, while all other population-based cohorts listed in Table 2 focus on the better

hearing ear. The Finnish twin study also compared hearing ability for the worse and better ear [75].

Table 2 Summary of epidemiological population based cohorts used to study on age-related hearing impairment

cohort	n	gender (% female)	age range mean(SD)	hearing tests	environmental and medical measures
The Framingham cohort [38,54,55,56]	1662	59%	63-95 73 (NA)	otoscopic examination pure-tone air (0.25;0.5;1;2;4;6;8 kHz) conduction audiometry word recognition self-reported HL	cardiovascular measures from the Framingham heart study
The Epidemiology of Hearing Loss Study Beaver Dam cohort [2,57,58,59,60,61,76]	3753	57.5%	48-92 65.8(NA)	otoscopic examination pure-tone air (0.25;0.5;1;2;3;4;6;8 kHz) and bone (0.5;4 kHz) conduction audiometry self-reported HL	Income Education (years) Occupation Occupational noise exposure noise exposure Smoking (never, past, current) Diabetes Cardiovascular diseases Cholesterol High density lipoprotein cholesterol Blood pressure (systolic/diastolic)

cohort	n	gender (% female)	age range mean(SD)	hearing tests	environmental and medical measures
Beaver Dam offspring cohort [58,61]	3285	54.6%	21-85 49.2(9.9)	otoscopic examination pure-tone air (0.5;1;2;3;4;6;8 kHz) and bone (0.5;4 kHz) conduction audiometry word recognition in quiet and competing message	Income Education (years) Occupation childhood water supply Occupational noise exposure noise exposure smoking (current , pack-years) Diabetes Cardiovascular diseases total serum cholesterol HDL serum cholesterol weekly exercise central retinal arteriolar equivalent intima-media thickness statin use ear infections ear surgery Meniere disease otosclerosis central retinal venular equivalent nonsteroidal anti-inflammatory Hypertension
The Blue Mountains Hearing Study [62,63,64]	2431	56.1%	55-99 67 (95% CI: 66.7-67.4)	pure-tone air (0.25;0.5;1;4;6;8 kHz) and bone conduction (if required) audiometry self-reported HL Hearing Handicap Inventory for the Elderly Screening Version	marital status ethnicity occupation occupational noise exposure ototoxic drug use hearing aid use 36-item short form health survey
Finnish twin study on ageing [73,74,75]	429	100%	63-76 NA	pure-tone air (0.125;0.25;0.5;1;2;4;8 kHz) conduction audiometry speech discrimination test self-reported HL	Mini-Mental State examination chronic diseases medication use smoking status Body Mass Index hearing aid ownership frequent otitis media otosclerosis auditory diseases acoustic trauma noise exposure (work or leisure) impulse work exposure

cohort	n	gender (% female)	age range mean(SD)	hearing tests	environmental and medical measures
ARHI study [65,67,68,69,70,71,72,77]	4083	51.8%	53-67 males:60.9 (3.2) females:60.4 (3.2)	pure-tone air (0.25;0.5;1;2;3;4;6;8 kHz) and bone (0.5;1;2;4 kHz) conduction audiometry	height weight Body Mass Index eye-colour left/right-handedness sunburn susceptibility hypertension whiplash injuries osteoporosis osteoarthritis allergy diabetes Heart attack cardiovascular diseases aspirin use atorvastatin use noise exposure (years) solvent exposure (years) occupational noise exposure exposure to gunfire smoking (current and past; pack-years) alcohol consumption

Data is based on the cohort profile as presented in the referenced publications. Data might vary in further studies on the cohorts. NA= information not available.

Environmental and medical risk factors

Most complex diseases, like ARHI, are caused by a combination of environmental and genetic risk factors, as well as the interaction of both. In addition, environmental factors can have an effect on epigenetic structures [78]. Genetic risk factors for ARHI are covered in chapter 5 and therefore not part of this introduction. Environmental risk factors for ARHI have been studied in various epidemiological population-based studies (Table 2). The environmental risk factors summarized in this paragraph are taken primarily from the cohorts studied in Table 2. Environmental risk factors for ARHI include noise exposure, smoking, alcohol consumption, ototoxic medication as well as medical conditions (i.e. cardiovascular diseases and diabetes mellitus). Each of the risk factors is covered in more detail below and significant associations summarised in Table 3.

Noise exposure above 85 dB A was reported to have the potential to cause HL [79]. However, experiments in mice revealed, that the time of noise exposure can affect the

intensity of HL and that exposure in young years is most destructive [80]. In the Beaver Dam cohort, occupational noise exposure had a significant effect on HL (HL was defined as a pure-tone average (0.5-4kHz) >25dB HL in the worse ear)(OR=1.31, 95% CI: 1.10-1.56)[81]. This association could be replicated in the Beaver Dam offspring cohort (Age-and sex-adjusted OR:1.67 (95% CI:1.29-2.16), multivariate OR:1.57 (95% CI: 1.19-2.08)[58]. In addition, individuals from the Beaver Dam cohort engaging in noisy leisure activities (≥90dB) were more likely to have HL than unexposed participants (OR:1.11, 95%CI: 1.01-1.22)[76].

In the ARHI study, occupational noise exposure was associated with Z-scores for the higher frequencies (2-8 kHz) ($p=1.0x10^{-17}$) for all European centres besides Antwerp [66]. The length of noise exposure in years was also significantly associated with ARHI in the ARHI study (Z-scores for the high frequencies, $p=1.0x10^{-17}$), however, an association between Gunfire noise exposure and Z-scores (p=0.009) did not survive the Bonferroni correction for 74 tests ($p<6.8x10^{-4}$)[66].

Cigarette smoking can lead to vasoconstriction and thus reduce blood supply to the cochlea. Smoking has further been predicted to have an effect on the antioxidant defence of the cochlea to reactive oxygen species. [82,83]. In the Beaver Dam cohort, current smokers had a 1.69 times higher risk of ARHI than non-smokers (OR: 1.69; 95%CI:1.31-2.17), this effect was even more pronounced in individuals with additional occupational noise exposure (OR:1.85; 95%CI: 1.33-2.57) and remained in smokers unexposed to noise (OR: 1.53; 95%CI: 1.03-2.29)[59]. In the Beaver Dam offspring cohort, Smoking more than 11 packs of cigarettes per year showed a significant effect on ARHI compared to non-smokers (OR:1.61; 95% CI:1.16-2.23) after adjustment for age and gender [58]. However, current smoking (having smoked more than 100 cigarettes) was not significantly associated with HL (OR: 1.40; 95% CI:0.99-1.98)[58]. This leads to the conclusion that a threshold of smoking needs to be reached to affect hearing ability. In the ARHI study, smoking history measured in packyears (packages of cigarettes smoked per year) had a significant effect on the Z-scores for the higher frequencies ($p=1.0x10^{-9}$) and remained significant for males ($p=1.9x10^{-7}$) when stratified by gender [66]. A significant effect of smoking on HL was seen after adjustment for cardiovascular diseases and body mass index ($p=2.4x10^{-8}$) [66]. Furthermore, a dosage effect within the group of current smokers could be shown for HL in the higher frequencies ($p=3.0x10^{-7}$) [66].

The effect of alcohol consumption on ARHI has been controversial. In the ARHI study, moderate alcohol consumption (at least one unit of alcohol per week) had a positive effect on ARHI, leading to a significant increase in hearing ability at the higher frequencies ($p=8.4x10^{-6}$)[66]. The same positive effect of moderate alcohol consumption on hearing ability had been reported previously for light drinkers in a Japanese Health screen cohort (OR=0.73, 95% CI: 0.56-0.94) after adjustment for age and sex as well as other risk factors for HL [84].

Kakarlapudi et al compared the prevalence of sensorineural HL between diabetic and healthy individuals and determined a higher prevalence for the diabetic group [85] (13.1% versus 10.3%, respectively, $p<0.05$). However, diabetes was neither significantly associated with ARHI in the ARHI study ($p=0.0636$) nor in the Beaver Dam cohort or the Beaver Dam offspring cohort (OR=1.21, 95% CI: 0.79-1.83)[57,58,77]. These results might have been biased by the low number of diabetic individuals in both Beaver Dam and ARHI study cohorts.

Torre et al [60] compared distortion product otoacoustic emissions of healthy individuals and subjects with a self-reported history of cardiovascular diseases. This test relies on the ability of outer hair cells to emit sound [86] and is generally applied as a functional test of the cochlea. It was shown that women with a history of myocardial infarction had an increased risk of HL compared to healthy women (OR=2.00, 95% CI: 1.15-3.46).

Aminoglycoside and macrolide antibiotics, loop diuretics, chemotherapeutic drugs and antimalarials are known to have an ARHI inducing effect. These therapeutic agents are therefore summarised as ototoxic medication. Intake of ototoxic medication can lead to outer hair cell death, damage to stria vascularis and cochlea in general, as well as degeneration of the organ of Corti [87].

Table 3 Summary of significant associations of environmental and medical risk factors with ARHI

risk factor (environmental or medical)	cohort [reference]	phenotype	ethnicity	n	sex M/F	age	OR (95% CI)	p-value
occupational noise exposure (yes/no)	Beaver Dam [81]	PTA (0.5-4kHz) >25dB HL	Caucasian (American)	3753	1589 2164	48-92	1.31 (1.10-1.56)	-
	Beaver Dam offspring [58]	PTA (0.5-4kHz) >25dB HL	Caucasian (American)	2837	1294 1543	21-84	1.67 (1.29-2.16)	-
	ARHI study [66]	Z_{high}	Caucasian (European)	4083	1967 2116	53-67	-	$1.0x10^{-17}$
leisure noise exposure (yes/no)	Beaver Dam [76]	PTA (0.5-4kHz) >25dB HL	Caucasian (American)	3571	1540 2031	48-92	1.11 (1.01-1.22)	-
current smoking	Beaver Dam [59]	PTA (0.5-4kHz) >25 dB HL	Caucasian (American)	3571	1540 2031	48-92	1.69 (1.31-2.17)	-
smoking (>11 packs/year)	Beaver Dam offspring [58]	PTA (0.5-4kHz) >25 dB HL	Caucasian (American)	2837	1294 1543	21-84	1.61 (1.16-2.23)	
smoking history (packyears)	ARHI study [51]	Z_{high}	Caucasian (European)	4083	1967 2116	53-67	-	$1.0x10^{-9}$
alcohol consumption (≥1 unit/week)	ARHI study [66]	Z_{high}	Caucasian (European)	4083	1967 2116	53-67	-	$8.4x10^{-6}$
alcohol consumption (light drinkers)	Japanese health screen cohort [84]	>40 dB at 4 kHz bilaterally	Asian	3303	2684 619	60-80	0.73 (0.56-0.94)	-
diabetes mellitus (Type 1 and 2 diabetes; yes/no)	Maryland VA patient database [85]	SNHL	Caucasian (American)	66036	-	-	-	<0.05
myocardial infarction (yes/no)	Beaver Dam [60]	DPOAE	Caucasian (American)	877	0 877	68.6 ±0.32	2.00 (1.15-3.46)	-

Environmental and medical risk factors are further specified by exposure assessment (yes/no; ever/never, or intensity of exposure, i.e. packyears). Associations with following phenotypes has been described: Z-score calculated for the high frequencies (Z_{high}), pure-tone averages (PTA; for 0.5, 1, 2 and 4 kHz) >25dB), pure-tone thresholds at 4 kHz ≤40 dB, as well as sensorineural HL (SNHL) and distortion product otoacoustic emission (DPOAE). Ethnicity, sample size (n), sex of samples (M=male, F=female) and the age range or mean age (with standard error) are given for each association, if specified. The probability of association is defined either by the odds ratio (OR) and respective 95% confidence interval (95%CI) or the respective p-value.

Ageing theories and the molecular biology of ageing

Ageing is defined as the biological process of growing old. It is often associated with an accumulation of deleterious biological mechanisms leading to frailty and increased risk of death. Individuals that grow old without obvious pathologies or live longer than the average life expectancy predicts for their population, are referred to as “healthy agers”.

Signs and therefore measures of ageing include reduced telomere length, accumulation of nuclear somatic mutations, increased mitochondrial mutations and corresponding reduced mitochondrial efficiency as well as damage to various cellular structures by reactive oxygen species (ROS) and decreased metabolism.

Telomere length is controlled by the enzyme telomerase, which is responsible for elongating nucleotide repeats at the telomere ends of the chromosomes and was first discovered in ciliate protozoa [88]. Telomeres are naturally shortened by each round of cell division unless telomerase reverses the shortage. Stem cells naturally produce telomerase, whereas mitotic cells show reduced telomerase expression or fail to express the enzyme completely [89]. Furthermore, nuclear somatic mutations slowly increase with each round of DNA replication. Mitochondria are the energy providers of the cell. Superoxide anions, hydrogen peroxide and hydroxyl radicals make up the reactive oxygen species in the body. They are produced as a side product of metabolism, due to the chemical reduction of oxygen [90].This reduction of oxygen occurs in the mitochondria. ROS, which accumulate over a lifetime, can cause damage to various cellular building stones, including proteins, lipids and nucleic acids as well as structures in the mitochondrion itself. The damage to nucleic acids can link increased ROS production to genome instability, another cause of ageing. Antioxidants scavenge ROS and can therefore prevent damage to cellular structures. Animal studies of ageing or longevity often focus on the effect of antioxidants by decreasing or increasing their expression, respectively.

Many candidate genes for longevity were first discovered in studies of model organisms with exceptional longevity. Studying ageing or healthy ageing traits in model organisms particularly has the advantage of shorter lifespan in these organisms compared to humans. Preferred model organisms for ageing traits include *Saccharomyces cerevisiae* (yeast), *Drosophila melanogaster* (fruit fly), *Caneorhabditis elegans* (roundworm) and rodents such as mice [91]. Important ageing models are the *Sir4-42* yeast mutant [92], the *daf-2* roundworm mutant [93], the fruit fly mutant Methuselah and the $p66^{sh}$ mouse mutant [94]. Model organisms helped to understand various public ageing processes, but should always be taken with caution if applied to humans.

Determining human ageing genes often involves the study of individuals with extreme ageing phenotypes, thus focus is laid either on healthy or fast agers. Studies on “healthy ageing” genes are often based on the hypothesis that long-living and healthy ageing subjects are naturally selected for longer survival by a specific gene pool. It has been shown that offspring from centenarians have a higher survival risk than offspring from less successful agers. Identifying these genes selecting for longer survival has been the aim of many healthy ageing studies in which the genetic variants of centenarians are compared to the ones of younger unrelated subjects [95,96]. In other

studies, individuals with specifically fast ageing disorders are selected [97]. Diseases leading to premature ageing include Progeria, Bloom's, Cockayne's and Werner's syndrome [98].

Epigenetic studies of young and older identical twins showed that epigenetic profiles change with age, adding to increased discordance in older monozygotic twin pairs [99]. Epigenetic changes could provide a connection between increased exposure to environmental risk factors and genetic predisposition of ageing. Recent research proposes that DNA methylation acts like an "epigenetic clock" of human ageing [100]. The relationship between epigenetic factors and ageing phenotypes will be further explored in chapter 7.

Twin studies and the biology of twinning

A twin is defined as one of two individuals born together at the same time and from the same mother. The occurrence of twins has always fascinated humans and still cannot be fully explained. The rate of twin pregnancies increased from 11.6 multiple births per 1000 maternities in 1990 to 15.7 in 2010 in England and Wales (Office for National Statistics as of November 2011). Possible reasons for this phenomenon are increased in-vitro fertilization (implantation of multiple embryos), as well as better nutrition, giving twin pregnancies a higher survival rate than in times of famine. Twins can arise from one or two fertilized oocytes, giving the difference between monozygotic and dizygotic (fraternal) twins. In case of monozygotic twins, both twins arise from the same blastocysts, whereas dizygotic twins arise from two different eggs fertilized at the same time by two different sperms. Dizygotic twins share on average half of the alleles, like normal siblings, just that they share more environmental exposures than other siblings. Whether monozygotic twins share amniotic sac and placenta depends on the time the blastocyst fission. In general, the later the blastocyst splits, the more is shared during the pregnancy (Figure 5). What triggers splitting of the blastocyst in MZ twinning is not known.

Monozygotic twins were long time believed to share all their genetic material; however exemptions to this rule are accumulating. Monozygotic twins are often referred to as identical, although discordance for numerous phenotypes has been recognized in monozygotic twin pairs [101,102,103]. There are various possible reasons for discordance in MZ twins. They include unequal splitting of the blastocyst, mosaicism, somatic mutations in chromosomal or mitochondrial genes and epigenetic changes,

including imprinting or X silencing in females (excellently reviewed by *Machin* [104]). Better documentation of placenta sharing and recording the number of amniotic sacs has been requested to differentiate between the different “intermediate” forms of MZ twins [104]. The use of twins in heritability, genetic and epigenetic studies will be explained in more detail in the following chapters.

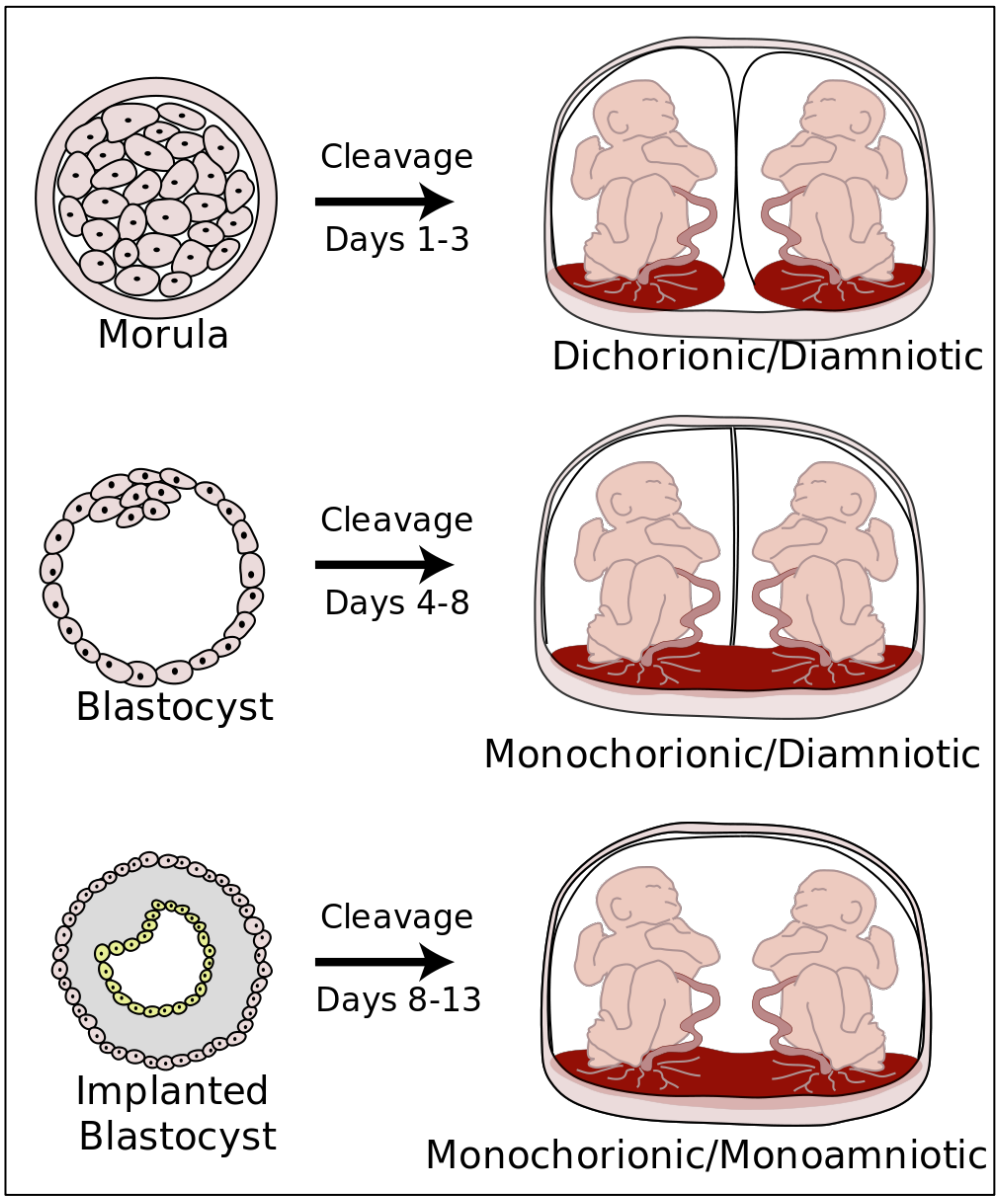


Figure 5 Different forms of monozygotic twins

(adapted from Dufendach, K. (2012) *Placentation*. Retrieved from http://commons.wikimedia.org/wiki/File:Placentation.svg).

Generalised and specific aims

ARHI is a common trait affecting large parts of the elderly population. Moderate to high heritability estimates have been determined for this heterogeneous disorder. Nevertheless, genetic investigations have yet failed to explain heritability estimates with current management of ARHI being limited to the use of digital hearing aids. Furthermore, methods to summarise and rate ARHI are highly variable between research groups with standardised methods highly required to facilitate comparison and collaboration. Specifically genetic epidemiological data on purely female cohorts of ARHI taking genetic, environmental and epigenetic factors into account is scarce. The study presented here aims to address this scarcity of research. We therefore defined following specific aims:

1. Determine the prevalence of ARHI and exposure to environmental risk factors of ARHI in older females from the United Kingdom.

2. Estimate the percentage of variance in hearing ability with age explained by genetic and environmental factors in accordance with the classical twin model.

3. Introduce a novel web-based speech in noise hearing test to measure hearing ability and evaluate this tests suitability to measure ARHI in comparison to the gold standard method of pure-tone audiometry.

4. Identify common genetic variants associated with hearing ability with age in a genome-wide association design using both pure-tone audiometry and speech-in-noise test results as measures of hearing phenotypes. Promising results from this analysis shall be followed up further.

5. Identify differentially methylated regions associated with hearing ability with age in an epigenome-wide association study. Furthermore, monozygotic twin siblings discordant for hearing ability with age will be analysed for regions differentially methylated between both twins associated with their phenotypic discordance.

References

1. Office for National Statistics (2009) National Projections 2009: UK population to exceed 65m by 2018. Office for National Statistics.

2. Dalton DS, Cruickshanks KJ, Klein BE, Klein R, Wiley TL, et al. (2003) The impact of hearing loss on quality of life in older adults. Gerontologist 43: 661-668.

3. Royal National Institute of the Deaf (2006) Facts and figures on deafness and tinnitus. RNID.

4. Marieb EN, Hoehn K (2007) Human anatomy & physiology: Pearson Education.

5. Graham J, Baguley D (2009) Ballantyne's Deafness: Wiley-Blackwell.

6. Bluestone CD (1996) Pathogenesis of otitis media: role of eustachian tube. The Pediatric Infectious Disease Journal 15: 281-291.

7. Waltner JG, Raymond S (1950) On the chemical composition of the human perilymph and endolymph. The Laryngoscope 60: 912-918.

8. Dallos P (1992) The active cochlea. The Journal of Neuroscience 12: 4575-4585.

9. Klang N, Liberman MC, Sewell WF, Guinan JJ (1986) Single unit clues to cochlear mechanisms. Hearing Research 22: 171-182.

10. Lavigne-Rebillard M, Pujol R (1986) Development of the auditory hair cell surface in human fetuses. Anatomy and embryology 174: 369-377.

11. Vollrath MA, Kwan KY, Corey DP (2007) The Micromachinery of Mechanotransduction in Hair Cells. Annual Review of Neuroscience 30: 339-365.

12. Palma FD, Holme RH, Bryda EC, Belyantseva IA, Pellegrino R, et al. (2001) Mutations in Cdh23, encoding a new type of cadherin, cause stereocilia disorganization in waltzer, the mouse model for Usher syndrome type 1D. Nat Genet 27: 103-107.

13. Friedman TB, Sellers JR, Avraham KB (1999) Unconventional myosins and the genetics of hearing loss. New York, NY, ETATS-UNIS: Wiley-Liss. 55 p.

14. Tilney LG, Derosier DJ, Mulroy MJ (1980) The organization of actin filaments in the stereocilia of cochlear hair cells. The Journal of Cell Biology 86: 244-259.

15. Crawford AC, Fettiplace R (1985) The mechanical properties of ciliary bundles of turtle cochlear hair cells. The Journal of Physiology 364: 359-379.

16. Probst FJ, Fridell RA, Raphael Y, Saunders TL, Wang A, et al. (1998) Correction of deafness in shaker-2 mice by an unconventional myosin in a BAC transgene. Science 280: 1444-1447.

17. Walsh T, Walsh V, Vreugde S, Hertzano R, Shahin H, et al. (2002) From flies' eyes to our ears: mutations in a human class III myosin cause progressive nonsyndromic hearing loss DFNB30. Proceedings of the National Academy of Sciences 99: 7518-7523.

18. Gibson F, Walsh J, Mburu P, Varela A, Brown K, et al. (1995) A type VII myosin encoded by the mouse deafness gene shaker-1. Nature 374: 62-64.

19. Wang A, Liang Y, Fridell RA, Probst FJ, Wilcox ER, et al. (1998) Association of Unconventional Myosin MYO15 Mutations with Human Nonsyndromic Deafness DFNB3. Science 280: 1447-1451.

20. Liu XZ, Walsh J, Mburu P, Kendrick-Jones J, Cope MJTV, et al. (1997) Mutations in the myosin VIIA gene cause non-syndromic recessive deafness. Nature Genetics 16: 188-190.

21. Berglund AM, Ryugo DK (1987) Hair cell innervation by spiral ganglion neurons in the mouse. The Journal of Comparative Neurology 255: 560-570.

22. Spoendlin H (1973) The innervation of the cochlear receptor. Basic mechanisms in hearing: 185-230.

23. Offner FF, Dallos P, Cheatham MA (1987) Positive endocochlear potential: Mechanism of production by marginal cells of stria vascularis. Hearing Research 29: 117-124.

24. Davis H (1965) A Model for Transducer Action in the Cochlea. Cold Spring Harbor Symposia on Quantitative Biology 30: 181-190.

25. Kikuchi T, Kimura RS, Paul DL, Adams JC (1995) Gap junctions in the rat cochlea: immunohistochemical and ultrastructural analysis. Anatomy and embryology 191: 101-118.

26. Dallos P, Evans BN (1995) High-Frequency Motility of Outer Hair Cells and the Cochlear Amplifier. Science 267: 2006-2009.

27. Zheng J, Shen W, He DZZ, Long KB, Madison LD, et al. (2000) Prestin is the motor protein of cochlear outer hair cells. Nature 405: 149-155.

28. Liberman MC, Gao J, He DZZ, Wu X, Jia S, et al. (2002) Prestin is required for electromotility of the outer hair cell and for the cochlear amplifier. Nature 419: 300-304.

29. Dallos P, He DZZ, Lin X, Sziklai I, Mehta S, et al. (1997) Acetylcholine, Outer Hair Cell Electromotility, and the Cochlear Amplifier. The Journal of Neuroscience 17: 2212-2226.

30. Rovers M, Haggard M, Gannon M, Koeppen-Schomerus G, Plomin R (2002) Heritability of symptom domains in otitis media: a longitudinal study of 1,373 twin pairs. American Journal of Epidemiology 155: 958-964.

31. Camp Gv, Smith R (2011) Hereditary Hearing Loss Homepage.

32. Van Laer L, Cryns K, Smith RJH, Van Camp G (2003) Nonsyndromic hearing loss. Ear and Hearing 24: 275-288.

33. World Health Organisation (2011) Grades of hearing impairment. In: Organization WH, editor. Prevention of Blindness and Deafness Geneva: World Health Organization.

34. AOHL (2011) Hearing matters. London: Action on Hearing Loss.

35. Davis A (1995) Hearing in adults: Whurr London.

36. Bedin E, Franzè A, Zadro C, Persico MG, Ciullo M, et al. (2009) Age-related hearing loss in four Italian genetic isolates: an epidemiological study. International Journal of Audiology 48: 465-472.

37. Gates GA, Mills JH (2005) Presbycusis. The Lancet 366: 1111-1120.

38. Gates GA, Couropmitree NN, Myers RH (1999) Genetic associations in age-related hearing thresholds. Arch Otolaryngol Head Neck Surg 125: 654-659.

39. Nolan LS, Maier H, Hermans-Borgmeyer I, Girotto G, Ecob R, et al. (2013) Estrogen-related receptor gamma and hearing function: evidence of a role in humans and mice. Neurobiology of Aging 34: 2077.e2071-2077.e2079.

40. World Health Organisation (2009) United Kingdom: general health statistical profile. WHO.

41. Schuknecht HF, Gacek MR (1993) Cochlear pathology in presbycusis. Ann Otol Rhinol Laryngol 102: 1-16.

42. Schuknecht HF (1964) Further observations on the pathology of presbycusis. Archives of Otolaryngology—Head & Neck Surgery 80: 369-382.

43. Liberman MC (1982) The cochlear frequency map for the cat: Labeling auditory‐nerve fibers of known characteristic frequency. The Journal of the Acoustical Society of America 72: 1441-1449.

44. Müller M (1991) Frequency representation in the rat cochlea. Hearing Research 51: 247-254.

45. Enander A, Stahle J (1967) Hearing in Menière's Disease: A Study of Pure-Tone Audiograms in 334 Patients. Acta Oto-Laryngologica 64: 543-556.

46. Nelson EG, Hinojosa R (2006) Presbycusis: a human temporal bone study of individuals with downward sloping audiometric patterns of hearing loss and review of the literature. The Laryngoscope 116: 1-12.

47. Sprinzl G, Riechelmann H (2010) Current trends in treating hearing loss in elderly people: a review of the technology and treatment options–a mini-review. Gerontology 56: 351-358.

48. Lotfi Y, Mehrkian S, Moossavi A, Faghih-Zadeh S (2009) Quality of life improvement in hearing-impaired elderly people after wearing a hearing aid. Arch Iran Med 12: 365-370.

49. Acar B, Yurekli MF, Babademez MA, Karabulut H, Karasen RM (2011) Effects of hearing aids on cognitive functions and depressive signs in elderly people. Archives of Gerontology and Geriatrics 52: 250-252.

50. Chatelin V, Kim EJ, Driscoll C, Larky J, Polite C, et al. (2004) Cochlear Implant Outcomes in the Elderly. Otology & Neurotology 25: 298-301.

51. Vermeire K, Brokx JP, Wuyts FL, Cochet E, Hofkens A, et al. (2005) Quality-of-life benefit from cochlear implantation in the elderly. Otology & Neurotology 26: 188-195.

52. Willott JF, Hnath Chisolm T, Lister JJ (2001) Modulation of presbycusis: current status and future directions. Audiology and Neurotology 6: 231-249.

53. Pujol R, Rebillard G, Puel JL, Lenoir M, Eybalin M, et al. (1991) Glutamate neurotoxicity in the cochlea: a possible consequence of ischaemic or anoxic conditions occurring in ageing. Acta Oto-Laryngologica 111: 32-36.

54. Dawber TR (1980) The Framingham Study: the epidemiology of atherosclerotic disease: Harvard university press Cambridge.

55. Gates GA, Cooper Jr J, Kannel WB, Miller NJ (1990) Hearing in the elderly: the Framingham cohort, 1983-1985. Part I. Basic audiometric test results. Ear and Hearing 11: 247.

56. DeStefano AL, Gates GA, Heard-Costa N, Myers RH, Baldwin CT (2003) Genomewide linkage analysis to presbycusis in the Framingham Heart Study. Arch Otolaryngol Head Neck Surg 129: 285-289.

57. Cruickshanks KJ, Wiley TL, Tweed TS, Klein BEK, Klein R, et al. (1998) Prevalence of Hearing Loss in Older Adults in Beaver Dam, Wisconsin: The Epidemiology of Hearing Loss Study. American Journal of Epidemiology 148: 879-886.

58. Nash SD, Cruickshanks KJ, Klein R, Klein BEK, Nieto FJ, et al. (2011) The prevalence of hearing impairment and associated risk factors: the Beaver Dam Offspring Study. Archives of Otolaryngology—Head & Neck Surgery 137: 432.

59. Cruickshanks Kj KRKBKWTLNDMTTS (1998) Cigarette smoking and hearing loss: The epidemiology of hearing loss study. JAMA 279: 1715-1719.

60. Torre P, III, Cruickshanks KJ, Klein BEK, Klein R, Nondahl DM (2005) The Association Between Cardiovascular Disease and Cochlear Function in Older Adults. J Speech Lang Hear Res 48: 473-481.

61. Zhan W, Cruickshanks KJ, Klein BEK, Klein R, Huang G-H, et al. (2010) Generational Differences in the Prevalence of Hearing Impairment in Older Adults. American Journal of Epidemiology 171: 260-266.

62. Sindhusake D, Mitchell P, Smith W, Golding M, Newall P, et al. (2001) Validation of self-reported hearing loss. The Blue Mountains hearing study. International Journal of Epidemiology 30: 1371-1378.

63. Gopinath B, Flood V, McMahon C, Burlutsky G, Smith W, et al. (2010) The effects of smoking and alcohol consumption on age-related hearing loss: The Blue Mountains Hearing Study. Ear and Hearing 31: 277.

64. Chia EM, Wang JJ, Rochtchina E, Cumming RR, Newall P, et al. (2007) Hearing impairment and health-related quality of life: the Blue Mountains Hearing Study. Ear and Hearing 28: 187-195.

65. Van Eyken E, Van Camp G, Van Laer L (2007) The complexity of age-related hearing impairment: contributing environmental and genetic factors. Audiology and Neurotology 12: 345-358.

66. Fransen E, Topsakal V, Hendrickx JJ, Van Laer L, Huyghe JR, et al. (2008) Occupational noise, smoking, and a high body mass index are risk factors for age-related hearing impairment and moderate alcohol consumption is protective: a European population-based multicenter study. JARO-Journal of the Association for Research in Otolaryngology 9: 264-276.

67. Van Laer L, Van Eyken E, Fransen E, Huyghe JR, Topsakal V, et al. (2008) The grainyhead like 2 gene (GRHL2), alias TFCP2L3, is associated with age-related hearing impairment. Hum Mol Genet 17: 159-169.

68. Van Eyken E, Van Camp G, Fransen E, Topsakal V, Hendrickx JJ, et al. (2007) Contribution of the N-acetyltransferase 2 polymorphism NAT2*6A to age-related hearing impairment. J Med Genet 44: 570-578.

69. Garringer HJ, Pankratz ND, Nichols WC, Reed T (2006) Hearing Impairment Susceptibility in Elderly Men and the DFNA18 Locus. Arch Otolaryngol Head Neck Surg 132: 506-510.

70. Huyghe JR, Van Laer L, Hendrickx JJ, Fransen E, Demeester K, et al. (2008) Genome-wide SNP-based linkage scan identifies a locus on 8q24 for an age-related hearing impairment trait. Am J Hum Genet 83: 401-407.

71. Friedman RA, Van Laer L, Huentelman MJ, Sheth SS, Van Eyken E, et al. (2009) GRM7 variants confer susceptibility to age-related hearing impairment. Hum Mol Genet 18: 785-796.

72. Fransen E, Van Laer L, Lemkens N, Caethoven G, Flothmann K, et al. (2004) A novel Z-score-based method to analyze candidate genes for age-related hearing impairment. Ear Hear 25: 133-141.

73. Kaprio J, Koskenvuo M (2002) Genetic and environmental factors in complex diseases: the older Finnish Twin Cohort. Twin Research 5: 358-365.

74. Viljanen A, Era P, Kaprio J, Pyykkö I, Koskenvuo M, et al. (2007) Genetic and Environmental Influences on Hearing in Older Women. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences 62: 447-452.

75. Viljanen A, Kaprio J, Pyykkö I, Sorri M, Kauppinen M, et al. (2007) Genetic and environmental influences on hearing at different frequencies separately for the better and worse hearing ear in older women. International Journal of Audiology 46: 772-779.

76. Dalton DS, Cruickshanks KJ, Wiley TL, Klein BEK, Klein R, et al. (2001) Association of Leisure-Time Noise Exposure and Hearing Loss: Asociación entre exposición a ruido durante el tiempo libre e hipoacusia. International Journal of Audiology 40: 1-9.

77. Fransen E, Topsakal V, Hendrickx JJ, Van Laer L, Huyghe JR, et al. (2008) Occupational noise, smoking, and a high body mass index are risk factors for age-related hearing impairment and moderate alcohol consumption is protective: a European population-based multicenter study. J Assoc Res Otolaryngol 9: 264-276; discussion 261-263.

78. Wong CCY, Caspi A, Williams B, Craig IW, Houts R, et al. (2010) A longitudinal study of epigenetic variation in twins. Epigenetics 5: 516-526.

79. NIH (1990) Noise and Hearing Loss: Consensus Statement, NIH Consensus Development Conference, January 22-24, 1990: U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Office of Medical Applications of Research.

80. Kujawa SG, Liberman MC (2006) Acceleration of age-related hearing loss by early noise exposure: evidence of a misspent youth. The Journal of Neuroscience 26: 2115-2123.

81. Cruickshanks KJ, Wiley TL, Tweed TS, Klein BE, Klein R, et al. (1998) Prevalence of hearing loss in older adults in Beaver Dam, Wisconsin. The Epidemiology of Hearing Loss Study. Am J Epidemiol 148: 879-886.

82. Maffei G, Miani P (1962) Experimental tobacco poisoning: Resultant structural modifications of the cochlea and tuba acustica. Archives of Otolaryngology—Head & Neck Surgery 75: 386.

83. Shapiro S (1964) ARE YOU SMOKING MORE BUT HEARING LESS? Eye, ear, nose & throat monthly 43: 96.

84. Itoh A, Nakashima T, Arao H, Wakai K, Tamakoshi A, et al. (2001) Smoking and drinking habits as risk factors for hearing loss in the elderly: epidemiological study of subjects undergoing routine health checks in Aichi, Japan. Public Health 115: 192-196.

85. Kakarlapudi V, Sawyer R, Staecker H (2003) The effect of diabetes on sensorineural hearing loss. Otol Neurotol 24: 382-386.

86. Kemp DT (1978) Stimulated acoustic emissions from within the human auditory system. The Journal of the Acoustical Society of America 64: 1386-1391.

87. Rybak LP, Ramkumar V (2007) Ototoxicity. Kidney Int 72: 931-935.

88. Greider CW, Blackburn EH (1985) Identification of a specific telomere terminal transferase activity in tetrahymena extracts. Cell 43: 405-413.

89. Wright WE, Piatyszek MA, Rainey WE, Byrd W, Shay JW (1996) Telomerase activity in human germline and embryonic tissues and cells. Developmental genetics 18: 173-179.

90. Harman D (1981) The aging process. Proceedings of the National Academy of Sciences 78: 7124-7128.

91. Guarente L, Kenyon C (2000) Genetic pathways that regulate ageing in model organisms. NATURE-LONDON-: 255-262.

92. Kennedy BK, Austriaco Jr NR, Zhang J, Guarente L (1995) Mutation in the silencing gene SIR4 can delay aging in S. cerevisiae. Cell 80: 485.

93. Kenyon C, Chang J, Gensch E, Rudner A, Tabtiang R (1993) A C. elegans mutant that lives twice as long as wild type. Nature 366: 461-464.

94. Migliaccio E, Giorgio M, Mele S, Pelicci G, Reboldi P, et al. (1999) The p66^ s^ h^ c adaptor protein controls oxidative stress response and life span in mammals. Nature 402: 309-312.

95. Atzmon G, Cho M, Cawthon RM, Budagov T, Katz M, et al. (2010) Evolution in health and medicine Sackler colloquium: Genetic variation in human telomerase is associated with telomere length in Ashkenazi centenarians. Proceedings of the National Academy of Sciences of the United States of America 107 Suppl 1: 1710-1717.

96. Gerdes LU, Jeune B, Ranberg KA, Nybo H, Vaupel JW (2000) Estimation of apolipoprotein E genotype-specific relative mortality risks from the distribution of genotypes in centenarians and middle-aged men: apolipoprotein E gene is a "frailty gene," not a "longevity gene". Genetic Epidemiology 19: 202-210.

97. Eriksson M, Brown WT, Gordon LB, Glynn MW, Singer J, et al. (2003) Recurrent de novo point mutations in lamin A cause Hutchinson-Gilford progeria syndrome. Nature 423: 293-298.

98. Dyer CAE, Sinclair AJ (1998) The premature ageing syndromes: insights into the ageing process. Age and Ageing 27: 73-80.

99. Fraga MF, Ballestar E, Paz MF, Ropero S, Setien F, et al. (2005) Epigenetic differences arise during the lifetime of monozygotic twins. Proceedings of the National Academy of Sciences of the United States of America 102: 10604-10609.

100. Horvath S (2013) DNA methylation age of human tissues and cell types. Genome biology 14: R115.

101. Kuratomi G, Iwamoto K, Bundo M, Kusumi I, Kato N, et al. (2007) Aberrant DNA methylation associated with bipolar disorder identified from discordant monozygotic twins. Molecular psychiatry 13: 429-441.

102. Baranzini SE, Mudge J, van Velkinburgh JC, Khankhanian P, Khrebtukova I, et al. (2010) Genome, epigenome and RNA sequences of monozygotic twins discordant for multiple sclerosis. Nature 464: 1351-1356.

103. Weksberg R, Shuman C, Caluseriu O, Smith AC, Fei Y-L, et al. (2002) Discordant KCNQ1OT1 imprinting in sets of monozygotic twins discordant for Beckwith–Wiedemann syndrome. Human Molecular Genetics 11: 1317-1325.

104. Machin G (2009) Non-identical monozygotic twins, intermediate twin types, zygosity testing, and the non-random nature of monozygotic twinning: A review. American Journal of Medical Genetics Part C: Seminars in Medical Genetics 151C: 110-127.

Chapter 2: Pure-tone audiogram data collection and phenotype definition

Abstract

In the Introduction various population samples for ARHI have been presented. Although ARHI is a highly prevalent disorder, methods to capture this multivariable trait differ between research groups and gold standards have not been completely defined. This chapter describes the methods used to obtain the hearing data in TwinsUK and data preparation for analysis.

Hearing ability was measured in volunteers from the TwinsUK cohort using the Madsen XETA screening audiometer and supra-aural headphones to obtain air-conduction pure-tone thresholds. In addition all participants were asked to complete an accompanying questionnaire covering self-reported hearing loss and exposure to environmental risk factors. Different methods were applied to summarise the multidimensional audiogram data (pure-tone average, better ear hearing level, principal component analysis) and compared in their suitability to capture and represent the characteristic features of ARHI. The specificity and sensitivity of self-reported hearing loss in comparison to pure-tone audiometry was evaluated and the effect of environmental exposure on hearing ability with age tested. Reproducibility of the audiogram was tested using a Bland-Altman comparison.

1309 females (mean age: 61.67 (±8.48) years) from the TwinsUK register completed both audiogram and hearing questionnaire. The first two principal components captured 70%-76% of the variation in pure-tone thresholds and represented the magnitude and shape of the audiogram, respectively. In contrast to averaging methods, principal components were able to capture not only the overall threshold shift but also the slope of the audiogram, characteristic for ARHI. In comparison to other samples, prevalence of slight (27.55%) and moderate (6.06%) hearing loss was relatively low in TwinsUK. Self reported hearing ability showed 87% sensitivity and 76% specificity to determine moderate hearing loss. Of the risk factors detected on the questionnaire, only reported otitis media during childhood had a significant effect on principal component 1 in a stepwise multivariate regression (beta± SE=1.13±0.49; p=0.023; 95%CI: 0.16-2.11)), whereas changes in principal component 2 were significantly associated with exposure to noisy handiwork (beta± SE=0.82± 0.23, p=0.001; 95% CI: 0.36-1.28) and occupational noise (beta± SE=0.74±0.37, p=0.050; 95% CI: 5.50×10^{-4}-

1.47). Reproducibility of the audiogram was assessed in 117 individuals that completed the test twice at different visits and found highly reproducible with a mean difference in pure-tone average of 2.082 dB HL (95% CI: -2.762 to -1.402) between both tests.

In conclusion, we report the collection of a new female population sample for ARHI of northern European ancestry. According to our comparison, principal component analysis was the most suitable method to capture ARHI, representing threshold shift and slope of the audiogram and including the results of older individuals. The hearing test applied in this cohort was highly repeatable and previously reported environmental risk factors for ARHI showed only minor affects on ARHI in TwinsUK volunteers.

Introduction

Basic acoustics

To understand human hearing ability and measurements of the same, a basic introduction into acoustics is given. Sounds are created by vibrations, which are transmitted through an elastic medium to the listener, who can detect and interpret the sound. In everyday hearing, the elastic medium transmitting the sound is air. Vibrations will be constantly repeated when traveling through this medium, resulting in a waveform like the one depicted in Figure 6.

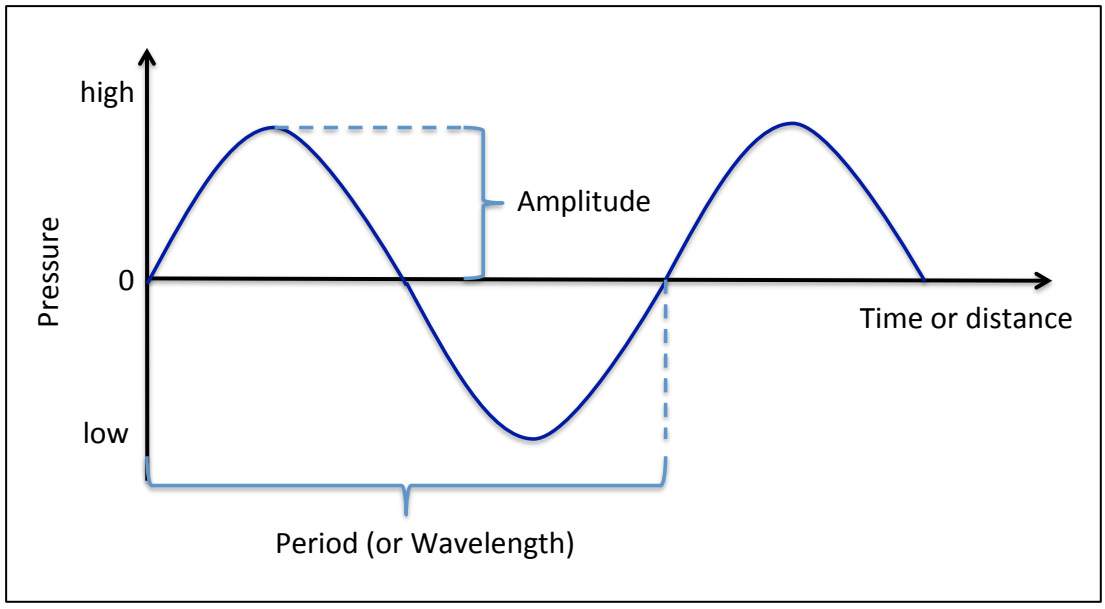


Figure 6 Waveform of a pure-tone (adapted from M. Tate Maltby)[1]

The waveform of a pure-tone is defined by its amplitude and period.

A single sinusoidal sound wave is referred to as a pure tone. It is defined by two major measures, frequency and intensity (or pressure level). The period or wavelength represents the time (or distance) required for the sound wave to complete one cycle. The frequency refers to the inverse of the period (frequency= 1/period) and is measured in hertz (Hz) with one Hz corresponding to one period per second (1Hz=1 period/second). The human ear is generally able to detect frequencies between 20 Hz and 20 kHz [1].

The sound intensity (or sound pressure level) corresponds to the amplitude of the sound wave. The amplitude is defined as the variation of the wave's pressure around its mean. Because an undisturbed acoustic wave always returns to its origin after completion of half a cycle and a full cycle, the mean pressure is calculated as 0. The amplitude of a sound wave determines how much compression (in case of high pressure) and rarefaction (low pressure) is enforced on the elastic medium it is traveling through, and is directly proportional to the intensity of the force having created the original vibration. A stronger vibration will therefore cause higher amplitude and thus increased sound intensity.

Sound intensity is measured in bels and expressed as a ratio between the measured sound intensity and a reference sound pressure. The sound pressure level or sound intensity required to hear a pure-tone at a specific frequency, is measured in decibel (one tenth of a bel). The sound pressure measured (P_m) for an individual is compared to a reference sound pressure level (P_{ref}) and expressed as a logarithm to the base 10, multiplied by 20 [2] (Equation 1)[1].

Sound pressure (dB HL)= $20 \text{Log}_{10}(P_m/P_{ref})$

[Equation 1] Sound pressure

If the lowest sound intensity an individual can hear is equal to the reference sound pressure, the hearing test would measure 0 decibel hearing level (dB HL). If P_m was tenfold larger than the reference pressure (P_{ref}), this would correspond to 20 dB HL. Sound intensity therefore increases tenfold in steps of 20 dB HL (i.e. 40 dB HL sound intensity is tenfold louder than 20 dB HL). However, the latter rule only applies for dB values measured against the same reference pressure level. The current reference zero for the dB HL scale at different frequencies is defined in BS EN ISO 389-9: 2009 [3].

Recommended hearing test procedure

The air-conduction pure-tone audiogram is accepted as the standard hearing test by most audiologists and measures the lowest sound intensity a person can detect for different frequencies, commonly ranging from 0.125 to 8.0 kHz. The sound pressure threshold required to hear a sound at a particular frequency in at least 50% of the time is referred to as pure-tone threshold (PTT) [1].

Prior to the hearing test, a short patient history should be obtained and an otoscopic examination conducted. Otoscopy will be conducted of the outer and border to the middle ear, focusing particularly on the ear canal and tympanic membrane. Abnormalities of the tympanic membrane, like rupture, inflammation, effusion of the middle ear, as well as excessive wax build up obstructing the ear canal, should be recorded. To obtain PTTs the patient is asked to sit in a quiet room (<35 dB A or ideally a sound isolated booth) equipped with headphones connected to an audiometer and a response button. The patient is asked to listen closely and to press the button upon hearing of the beep sound. The tester should stay in visual contact with the subject to be tested at all times. The patient should not be able to determine when a beep sound had been send other than by hearing. For further detail on the test procedure, the standard procedures for pure-tone audiometry [4] published by the British Society of Audiology should be referred to.

Advantages and disadvantages of pure-tone audiometry

The audiogram gives audiologists the advantage to measure an individual's hearing ability for different frequencies and both ears separately, giving a detailed picture of a person's hearing level and allowing possible hearing disorders to be detected and diagnosed. If a bone-conduction pure-tone audiogram is performed, this can further help to differentiate between sensorineural, conductive or mixed types of hearing loss (HL).

The air-conduction audiogram allows making conclusions, although limited, about underlying pathologies and the impact of the hearing disorder on a subject's everyday life. Whereas vowels are usually located in the lower frequency spectrum (0.125 to 1.0 kHz), consonants are pronounced in the higher frequencies (1.0 to 8.0 kHz). HL in the higher frequencies, which are usually affected first in ARHI, makes it difficult to differentiate between consonants. According to Schuknecht [5], the pure-tone audiogram can predict different cochlear pathologies in respect to ARHI (chapter 1). Nevertheless, audiometry relies on hearing pitch sounds rather than words or full

sentences. Therefore disorders in speech processing and word discrimination due to auditory neuropathy cannot be excluded by the means of this test. Furthermore, dead regions in the cochlea might be missed by the pure-tone audiogram [6].

Although the pure-tone audiogram gives a detailed impression of a person's hearing ability; it creates a multitude of data. Therefore, a suitable method is required to summarize the multidimensional audiogram data and reduce the number of variables. A first step in this reduction method is to select test results for only one ear per person. A normal hearing person is assumed to show similar hearing sensitivity in both ears. Asymmetrical HL affecting only one of both ears is often caused by environmental exposure or distinct pathologies affecting only this ear (i.e. due to a vestibular schwannoma). In most analyses, the better hearing ear is selected, assuming that this ear reflects normal hearing ability and reducing bias due to differential environmental exposure between both ears at the same time. Further summary methods include taking the average of all pure-tone thresholds or only selected pure-tone thresholds [7]. In addition, it is common practice to standardise the pure-tone audiogram against pure-tone thresholds expected for a normal hearing person of the respective age and sex, thus generating standardized Z-Scores [8]. A recently introduced method summarises pure-tone thresholds by calculation of principal components [9]. In studies on ARHI all three different summary methods are currently applied. The lack of a gold-standard method impairs the comparison between different studies, therefore having a major impact on research in the field.

Materials and Methods

Sample recruitment

Volunteers were recruited from the TwinsUK registry located at St. Thomas hospital, London. The twin registry was created in 1992, and has since recruited mainly female same sex volunteer twin pairs. Hearing data, in form of a screening air-conduction pure-tone audiogram without masking, was collected as part of various studies between 2009 and 2013. Initial recruitment included all female twin pairs (both monozygotic and dizygotic) over the age of 40. Individuals affected by deafness or reporting early onset (<18 years) HL were excluded from the recruitment. All research was conducted according to the ethical standards as defined by the Helsinki declaration. Ethical approval for this study was obtained from the National Research Ethics service London-Westminster (REC reference#: 07/H0802/84). Written informed consent was obtained from all participants prior to study conduction. Each volunteer

was assigned a unique identification number, which could not be associated with the individual by the researcher, thereby ensuring confidentiality of medical and demographic data. The hearing test and hearing questionnaire were set up by Claire J. Steves as part of the TwinsUK Cogageing study, investigating the epidemiology of cognitive function in the elderly, and was later continued as part of other studies conducted in TwinsUK.

Pure-tone audiogram procedure

Air-conduction pure-tone audiometry was performed according to the procedures recommended by the British Society of Audiology [4]. All pure-tone audiograms were performed using a Madsen XETA screening audiometer equipped with supra-aural TDH39 headphones. Hearing test equipment was re-calibrated on a regular basis. Each hearing test was preceded by an otoscopic examination, recording abnormalities of the ear canal and tympanic membrane, as well as exceeding wax build up in subjects' ears. Participants were prepared for the test, explaining the test procedure according to a protocol developed by Claire Steves and asked whether they would have the energy to perform a hearing test taking about 15 minutes. Subjects were asked whether they experienced current hearing disorders (i.e. tinnitus, recent HL, infections or pain of the ear). The test was started on the better hearing ear as determined by participant self-report. If no better ear was reported, the test was started on the right ear. Pure-tone thresholds were measured for 8 frequencies in the following order: 1000 Hz, 2000 Hz, 4000 Hz, 6000 Hz, 8000 Hz, 1000Hz (retest), 500 Hz, 250 Hz and 125 Hz. The same procedure was used for the second ear without any pause between both ears. All hearing tests were performed on an automated basis to limit bias by tester variation.

The first pure-tone signal was presented at a well audible frequency (40 dB) for a normal hearing individual. In case of a positive reply (i.e. the participant heard the signal and pressed the response button), the sound intensity was lowered in 10 dB steps until no response indicated that the sound was inaudible. From the first inaudible threshold, the sound intensity was increased in steps of 5 dB until a positive response occurred, thus applying a "10-down, 5-up" technique. The pure-tone threshold was defined as the sound intensity at which 2 to 4 (≥ 50 %) out of 4 signal presentations were heard. The research assistant taking the test remained quietly in the hearing test room with the participant at all times to reinstruct the participant if necessary. Volunteers were seated facing away from the audiometer throughout the test. Ambient noise levels were monitored on a regular basis using a sound-meter.

Hearing questionnaire

All participants completed a detailed questionnaire on medical and environmental risk factors relevant for hearing. This hearing questionnaire was designed under consideration of the. NoiseScan [10] and MRC ENT questionnaire created in an initial collaboration with the Finnish Twin study on Aging and Prof. Dai Williams aiming to streamline the questionnaire data and audiometry. The questionnaire covered self reported hearing ability; occurrence of ear infections; operations on the ears; exposure to leisure noise, explosions or gunfire with immediate HL and tinnitus; occupational noise and length of exposure; occupation as well as hearing aid usage. Questions on previous ear operations could be further specified for the right or left ear. Leisure noise was further divided into “loud music”; “noisy handiwork/power tools” and “gunshots”. Occupational options included “Professional or managerial”; “Non-manual or clerical”; “Manual”; Housewife”; “Student” or “None”. If a participant selected two occupations, she was asked to select the occupation she worked at for a longer time. Options for occupational noise exposure included: “No, never”, “Yes, for less than a year”, “Yes, for 1-5 years” and “Yes, for more than 5 years”. All other questions could be answered with no, yes or not known. A copy of the applied questionnaire can be found in Appendix chapter 2.1.

Initial quality control

To reduce heterogeneity and HL due to other pathologies than ARHI, individuals with incomplete pure-tone audiogram data (>1 missing pure-tone threshold in one of both ears), who did not fit into the specified target group (females ≥40 years of age) or had reported a previous history of hereditary ear diseases and/or specific surgery on their ears (i.e. Cholesteatoma, Mastoiditis, Ossicle operations or Otosclerosis) were excluded from further analysis. In the case that a volunteer completed the hearing test twice (i.e. at different visits between 2009 and 2013) the test data collected at the later date was selected.

Calculation of pure-tone audiogram phenotypes

There is no gold standard definition for measuring ARHI and different research groups have used pure tone averages (PTAs) for different frequency ranges, standardized Z-scores [8], a better ear hearing level threshold (BEHL) [7] or principal component (PC) scores calculated in a principal component analysis [9] to summarise hearing ability measured by pure-tone audiometry. Most pure-tone audiogram phenotypes are calculated for the better ear of each subject. The better ear was defined as the ear with the lower PTA, calculated over all measured frequencies.

Pure-tone averages: PTA and BEHL

The PTA and BEHL are averages calculated from pure-tone thresholds (PTTs) over all frequencies or frequencies 0.5 kHz-4.0 kHz for the better ear, respectively. Both measures differ in the frequencies at which PTTs are measured. The choice of PTA or BEHL thus depends on availability of different frequency specific measures. PTA and BEHL values were calculated according to following equations:

$$PTA = \frac{(PTT_{0.125} + PTT_{0.25} + PTT_{0.5} + PTT_{1.0} + PTT_{2.0} + PTT_{4.0} + PTT_{6.0} + PTT_{8.0})}{8}$$

[Equation 2] Pure-tone average over all frequencies (0.125-8.0 kHz)
With PTT_x= pure-tone threshold at frequency x.

$$BEHL = \frac{(PTT_{0.5} + PTT_{1.0} + PTT_{2.0} + PTT_{4.0})}{4}$$

[Equation 3] Better ear hearing level threshold
With PTT_x= pure-tone threshold at frequency x.

Principal component analysis

Principal component analysis (PCA) is a statistical procedure to search for correlations in the multidimensional audiogram data, which are then summarized by new uncorrelated variables, called PCs. For the PCA performed here, PTTs measured for the eight covered frequencies were treated as eight correlated dimensions. PCs rely on correlations between these dimensions; therefore a correlation matrix for all dimensions is required. From this square correlation matrix, eigenvectors can be calculated. An eigenvector is defined as a vector, which can be multiplied by the respective matrix, without changing its direction. Eigenvectors are specific to a certain matrix and each square matrix can possess as many eigenvectors as columns (or rows, respectively), if any. All eigenvectors of one matrix are perpendicular to one another with their directions resembling correlations between the dimensions. Each eigenvector has its own eigenvalue, which represents the factor change in magnitude the eigenvector experiences by multiplication with the matrix. An eigenvector with an eigenvalue ≥1 is considered to be important in the context of PCA [11].

PCA assumes normally distributed input data. To transform PTTs to standard normal, a constant of 20 (to achieve positive thresholds) was added and the logarithm to the base 10 taken of the resulting sum.

$$PTTnorm = \log_{10}(PTT + 20)$$

[Equation 4] log-transformation of pure-tone-thresholds
With *PTT*=pure-tone threshold

Two principal component analyses were performed. The first analysis (PCA) was based on the above-described log-transformed pure-tone thresholds. For the second PCA (age-adjusted PCA) each log-transformed pure-tone threshold was adjusted for chronological age of subjects at hearing test using linear regression analysis and residuals taken. The age-adjusted PCA was performed on age-adjusted residuals of the log-transformed pure-tone thresholds (Equation 4).

Z-scores

The calculation of hearing Z-scores has been described previously [8]. In brief, standard hearing thresholds according to the ISO 7029 standards are given for 18-70 year-olds. Pure-tone thresholds determined for an individual can therefore be compared to the standardized medians for the respective age and gender. To determine whether a person has better hearing ability than the average and how far his or her hearing ability deviates from the standard median, the distance in standard deviations is calculated. According to the ISO 7029, standard deviations of the distribution around the median differed depending on being above or below the median. The Z-score is defined as the difference of the measured pure-tone threshold to the standard median in standard deviations. As the standard deviations differed dependent on the whether the threshold was higher or lower than the standard, the final Z-score depends on which standard deviation is chosen.

It was decided not to calculate Z-scores for this study due to various reasons addressed in the discussion. Description of the Z-score method to summarise PTTs was added for completeness.

PTAs, the BEHL and PCs were calculated for all subjects. Values for the different zygosity groups (monozygotic twins (MZs), dizygotic twins (DZs) and unpaired twins) were compared in an analysis of variance (ANOVA).

PC1 and PC2 outlier exclusion

After calculation of PC1 and PC2 values for all participants (n=1309) the dataset was examined for individuals whose hearing ability deviated more than 3 standard deviations from the mean for either PC1 or PC2. Volunteers that fulfilled the described criteria were rated as statistical outliers and their pure-tone audiograms for the better ear were visually inspected for structures indicating underlying pathologies other than ARHI.

Comparison of pure-tone audiogram results to self-reported HL

As part of the questionnaire, all volunteers were asked to self-assess current hearing difficulties. This information was used to determine the sensitivity and specificity of self-reported HL compared to HL as measured by pure-tone audiometry. Self-reported HL was compared to mild (PTA≥25dB HL) and moderate HL (PTA≥40 dB HL) according to WHO guidelines [12]. Sensitivity was defined as the proportion of individuals with slight or moderate HL who correctly self-reported HL (Equation 5), while specificity represented the proportion of volunteers with normal hearing who correctly identified themselves with as having normal hearing (Equation 6)[13].

$$Sensitivity = \frac{TP}{TP + FN}$$

[Equation 5] Sensitivity

$$Specificty = \frac{TN}{TN + FP}$$

[Equation 6] Specificity

With *TP*=true positives, *FN*=false negatives, *TN*=true negatives and *FP*=false positives.

In addition, the general prevalence of slight and moderate HL in the TwinsUK cohort was determined according to the grades of hearing impairment published by the WHO [12].

Association of hearing ability with age and environmental risk factors

The relationship between measures of hearing ability and chronological age of the subjects was assessed by correlation analysis. Furthermore, the effect of environmental risk factors, as recorded in the questionnaire, on hearing ability was determined. Hearing ability, as measured by age-adjusted PC1 and PC2, was compared between individuals exposed or unexposed to the respective risk factors in a Student's t-test assuming unequal variances between samples (Welch's t-test)[14]. The t-test compares the means of a variable for two independent groups (subjects exposed versus unexposed to risk factor) in ratio to the standard error of the difference. Under the null hypothesis of no difference, sample means for both groups are expected to be similar. For categorical environmental exposure variables (i.e. occupational noise exposure and occupation) an analysis of variance (ANOVA) was conducted. In addition, summary tables and box-plots of age-adjusted PC1 and PC2 values for exposed and unexposed individuals were generated (Appendix chapter 2.2-2.12). It

was hypothesized that certain risk factors might not be independent of each other (i.e. exposure to chronic otitis media might be treated by insertion of a tympanostomy tube (i.e. eardrum-operation)). To test for dependence of risk factors, a factor analysis was performed. Furthermore, a stepwise generalized least squares regression with age-adjusted PC1 and PC2 values as dependent variable and risk factors as predictors was conducted adjusted for twin relatedness. In this regression, predictor variables are stepwise removed if they fail to show a significant effect on the dependent variable. A new model is fitted each time after discarding a covariate. The final model contained only independent predictors having a significant effect on hearing ability (age-adjusted PC1 or PC2, respectively).

Hearing test reproducibility

A Bland Altman comparison [15] was applied to evaluate the agreement between repeated hearing tests measures in individuals that completed the pure-tone audiogram twice at different study visits. In this comparison [15] the difference between the two measurements was plotted versus the respective mean between repeated measures. When using the Bland Altman plot for measuring test repeatability, the differences between both visits would be expected to cluster around the zero line, assuming no or only minor deterioration in hearing ability between both visits.

Results

Initial quality control

Pure-tone audiogram and questionnaire data were obtained for 1447 individuals. 79 Individuals were excluded due to missing pure-tone thresholds. For this study only subjects over the age of 40 were included, thus 24 individuals who were younger than this were excluded. Further, 12 male volunteers were rejected for this data collection. In addition, 23 individuals were excluded due to self-report of hereditary middle ear diseases and operations that could result in conductive HL. After the exclusion of 138 individuals due to initial quality control measures, 1309 individuals with pure-tone audiogram and questionnaire data remained for analysis.

Measures of hearing ability

Hearing ability was measured for both ears and the better hearing ear defined as that having a lower PTA. In 653 individuals, hearing ability was better in the left ear, while in 580 participants the right ear was better. Equal hearing ability for both ears was reported for 76 participants and the mean difference in PTA between both ears for all subjects was measured as $m(\Delta)\pm SD=4.811\pm 5.938$ dB HL. According to the BSA

standard procedures for pure-tone audiometry [4] a difference of ≤5 dB HL between tests ("retest value") does not represent a significant difference to warrant repetition of the hearing test. The PTTs for each frequency followed a positively skewed distribution, close to a lognormal distribution. After log-transformation, PTTs at all frequencies approached a normal distribution. Mean PTTs were raised for the higher frequencies and increased from 1 kHz to 8 kHz, with the lowest mean PTTs measured at 1kHz ($m(PTT_{1.0})$=15.700±9.995 SD dB HL) (Table 4). Hearing ability was most variable at the higher frequencies, which also showed an increased correlation with age (Table 4).

Different methods were used to summarize hearing ability as measured by pure-tone audiometry. First, methods that summarise the audiogram by taking the average of specific PTTs were compared. The average calculated over all frequencies (PTA) was slightly higher than the BEHL and had a slightly larger standard deviation (SD). When calculating separate PTAs for the low (0.125-0.5 kHz), medium (1.0-2.0 kHz) and higher (4.0-6.0 kHz) frequencies, thresholds for the mid-frequencies were lowest. These results reflect the structure of the pure-tone thresholds, as the lowest mean pure-tone thresholds were determined for frequencies 1 and 2 kHz.

Association with age increased for the higher frequencies and therefore higher for summary measures that took into account these frequencies (i.e. PTA, PTA(high) and PC1 unadjusted). PC2 values for the unadjusted PCA were negatively correlated with age. PCs calculated from age-adjusted PTTs showed a very low correlation with age, due to previous age-adjustment. In addition, one should consider PC1 and PC2 together when examining the correlation with age, as these two components represent two dimensions from the same dataset.

Table 4 Description of summary measures of hearing for the better ear

variable	n	mean	SD	min	max	r(age)
$PTT_{0.125}$	1309	18.0596	9.2606	-5	70	0.2267
$PTT_{0.25}$	1309	21.4278	8.6099	0	70	0.2022
$PTT_{0.5}$	1309	20.8060	8.8556	0	70	0.2759
$PTT_{1.0}$	1309	15.6998	9.9501	-5	70	0.3885
$PTT_{2.0}$	1309	16.1108	12.9682	-5	90	0.4459
$PTT_{4.0}$	1309	23.0932	16.0762	-10	100	0.5003
$PTT_{6.0}$	1309	29.0947	18.7267	-5	100	0.5121
$PTT_{8.0}$	1309	35.7601	23.1034	-5	120	0.5606
PTA	1309	22.5065	10.3207	1.25	77.5	0.5634
BEHL	1309	18.9274	9.9508	-1.25	73.75	0.5058
PTA(low)	1309	20.0978	8.1895	0	70	0.2558
PTA(medium)	1309	15.9053	10.5863	-5	75	0.4556
PTA(high)	1309	29.3160	17.7638	-6.6667	100	0.5739
PC1 unadjusted	1309	0.0340	2.0761	-6.6939	8.8899	0.5322
PC2 unadjusted	1309	-0.0285	1.3059	-4.8956	3.6691	-0.2694
PC1 age-adjusted	1309	-0.0258	1.9388	-5.8835	8.6539	0.0415
PC2 age-adjusted	1309	0.0054	1.3618	-4.9111	5.4643	-0.0114

Summary measures of hearing impairment are presented as mean value for all 1309 individuals (n) and standard deviation from the mean (SD). In addition, minimal (min) and maximal (max) values for each summary measure and the Pearson correlation coefficient (r(age)) for the association of the respective measure with age are presented. As summary measures of hearing impairment pure-tone thresholds (PTTs) for the better ear for different frequencies ($PTT_{0.125}$- $PTT_{8.0}$), the pure-tone average (PTA), the better ear hearing level (BEHL), pure-tone averages for different frequency ranges (PTA(low; medium; high)) and principal components from adjusted and unadjusted PTTs are given.

Description of principal components

Principal components were obtained from two sets of PTTs, log-transformed unadjusted and log-transformed age-adjusted PTTs (0.125- 8 kHz). In both PCAs, only the first two PCs had eigenvalues ≥ 1. Together, PC1 and PC2 explained 70.30% (Table 5) and 75.98% (Table 7) of the variance in pure-tone thresholds for the age-adjusted and unadjusted PCA, respectively. From the eigenvector loadings, hypotheses could be made about the dimensions the PCs represented. For both PCAs, eigenvector loadings followed similar directions for PC1 (Table 6 and Figure 7; Table 8 and Figure 8). Eigenvector loadings for PC1 were of the similar direction and magnitude for all frequencies, whereas eigenvector loadings for the unadjusted and age-adjusted PC2 were of opposite direction (Figure 7, Figure 8). However, PC2

loadings changed direction from the lower to the higher frequencies for both PC2s (Figure 7, Figure 8).

Table 5 Summary measures of the age-adjusted principal component analysis

PCs	Eigenvalue	Proportion of variance explained	Cumulative proportion of variance explained
PC1	3.7786	0.4723	0.4723
PC2	1.8454	0.2307	0.7030

Principal components (PCs) were obtained from age-adjusted log-transformed PTT (125-8.0 kHz) residuals. Eigenvalues and the proportion of variance in PTTs accounted for by PCs are listed.

Table 6 Eigenvector loadings for age-adjusted principal components

PCs	Frequencies (kHz)							
	0.125	0.25	0.5	1.0	2.0	4.0	6.0	8.0
PC1	0.3560	0.3733	0.3933	0.3936	0.3556	0.3385	0.3201	0.2843
PC2	-0.3932	-0.4093	-0.3217	-0.1055	0.1984	0.3652	0.4312	0.4523

The eigenvector loadings for PC1 and PC2 are listed for pure-tone thresholds at frequencies 0.125-8.0 kHz.

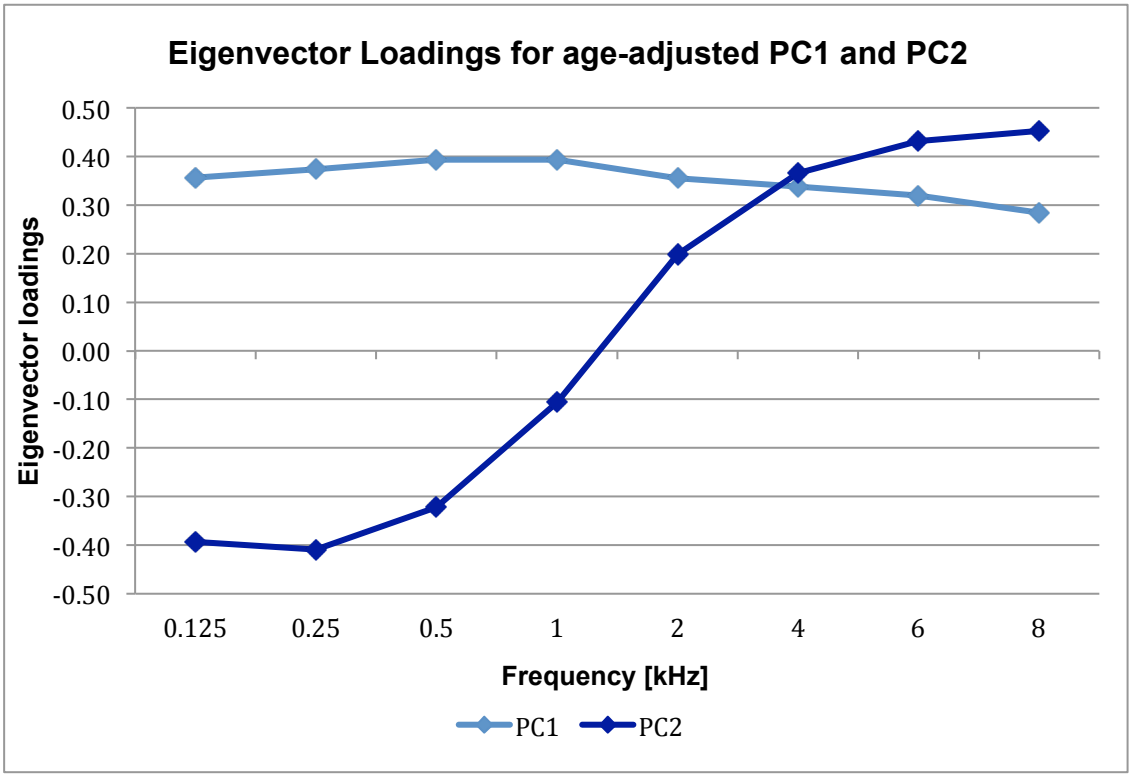


Figure 7 Eigenvector Loadings for age-adjusted PC1 and PC2

Eigenvector loadings as listed in Table 6 for age-adjusted PC1 and PC2 were plotted for the different frequencies.

Table 7 Summary measures of the unadjusted principal component analysis

PCs	Eigenvalue	Proportion of variance explained	Cumulative proportion of variance explained
PC1	4.3381	0.5423	0.5423
PC2	1.7404	0.2176	0.7599

Principal components (PCs) were obtained from log-transformed PTTs (125-8.0 kHz). Eigenvalues and the proportion of variance in PTTs accounted for by PCs are listed.

Table 8 Eigenvector loadings for unadjusted principal components

PCs	Frequencies (kHz)							
	0.125	0.25	0.5	1	2	4	6	8
PC1	0.3264	0.3313	0.3621	0.3833	0.3683	0.3643	0.3535	0.3352
PC2	0.4396	0.4718	0.3518	0.0914	-0.2022	-0.3329	-0.3751	-0.3995

The eigenvector loadings for PC1 and PC2 are listed for pure-tone thresholds at frequencies 0.125-8.0 kHz

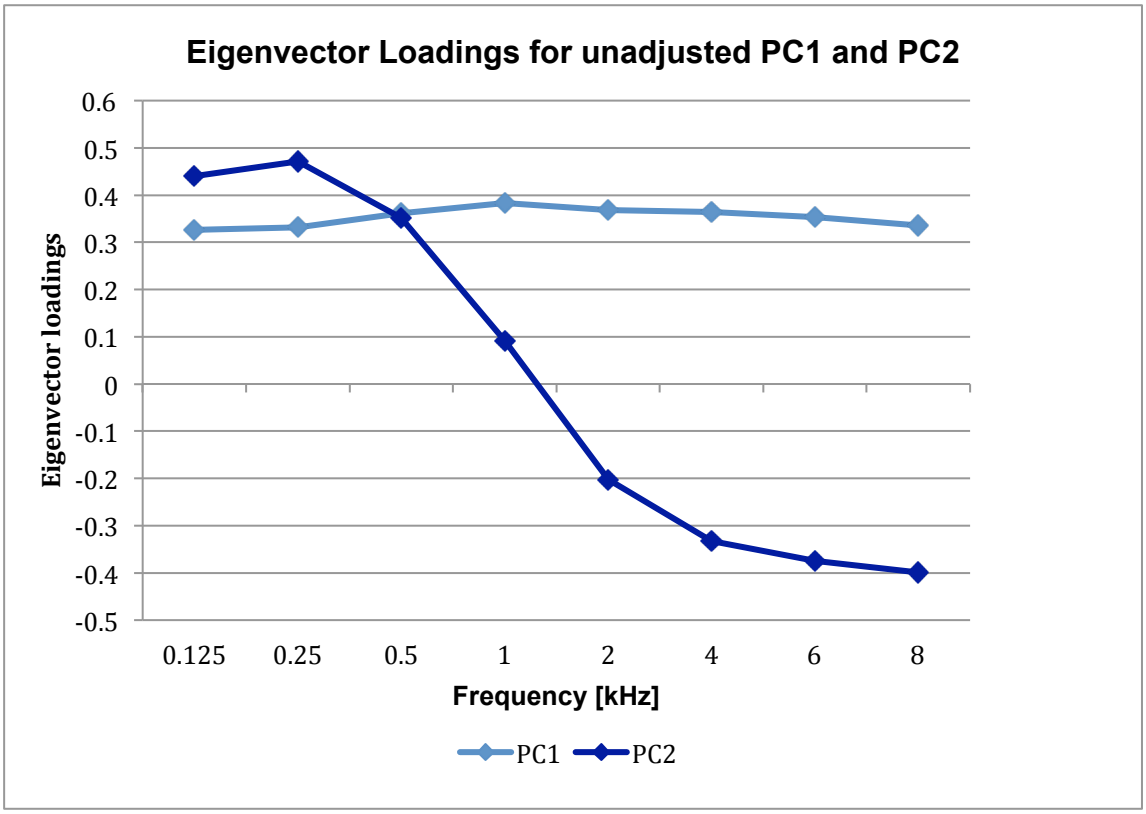


Figure 8 Eigenvector Loadings for unadjusted PC1and PC2

Eigenvector loadings as listed in Table 8 for unadjusted PC1 and PC2 were plotted for the different frequencies.

To understand better the meaning of PC1 and PC2 for hearing impairment, the dataset was divided into three equally sized groups (n= 436-437) depending on PC1 and PC2 values, respectively. For each group, the mean PTT (± standard error) at each frequency was plotted, resulting in a mean audiogram per group (Figure 9 and Figure 10). Individuals with a high PC1 value showed increased pure-tone thresholds over all frequencies, compared to individuals with lower PC1 values.

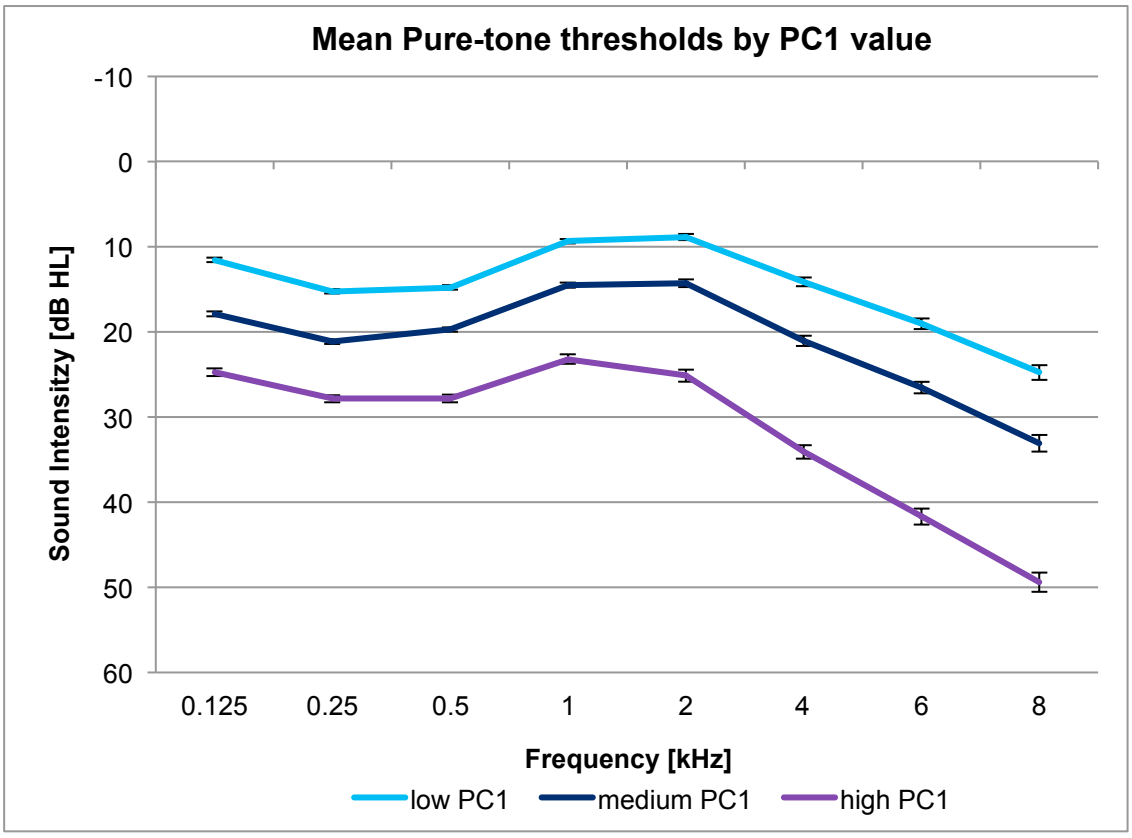


Figure 9 Pure-tone thresholds increase with increasing PC1 value

Subjects were divided into three groups according to their age-adjusted PC1 values (low, medium and high). For each group average PTTs per frequency were obtained and plotted as average pure-tone audiogram per group. Error bars represent the standard error of the mean PTTs.

In case of PC2, participants with a high PC2 value showed raised pure-tone thresholds particularly for the higher frequencies. This characteristic downslope in the audiogram for the higher frequencies could not be seen for the lower PC2 values, in which case the mean audiograms appeared flatter.

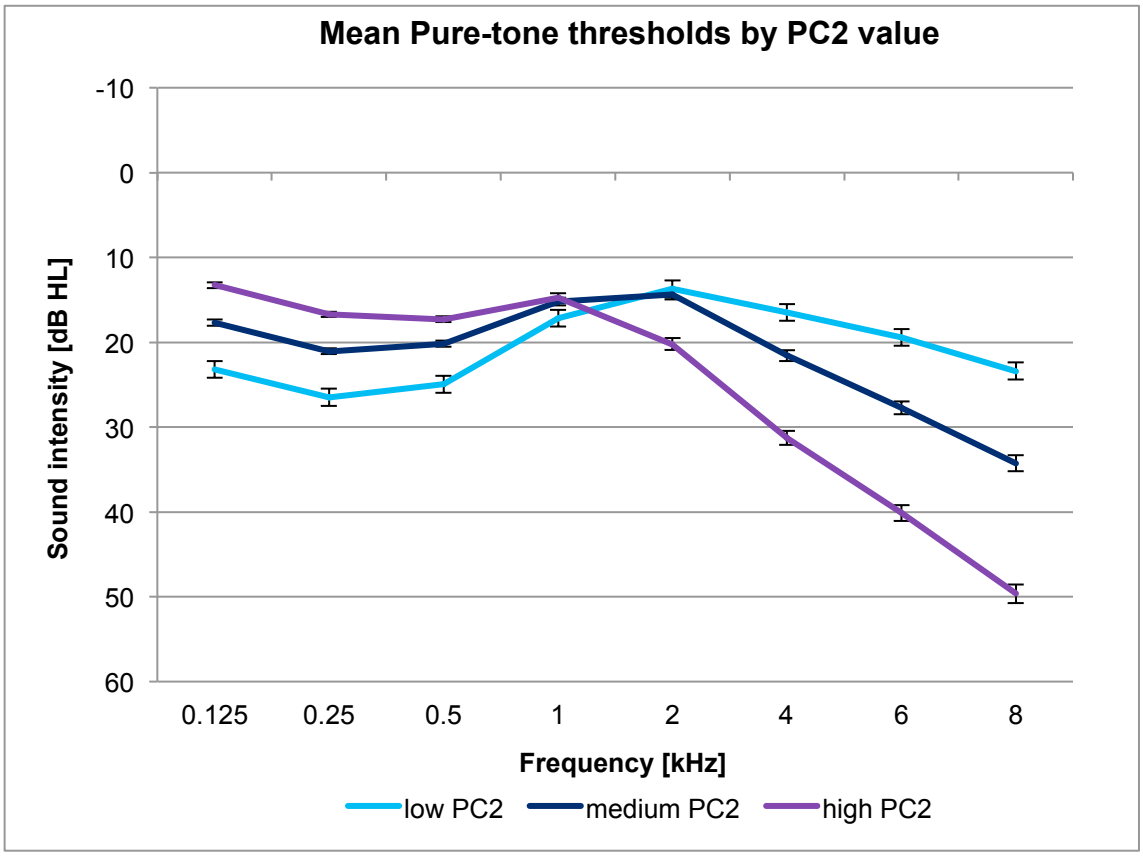
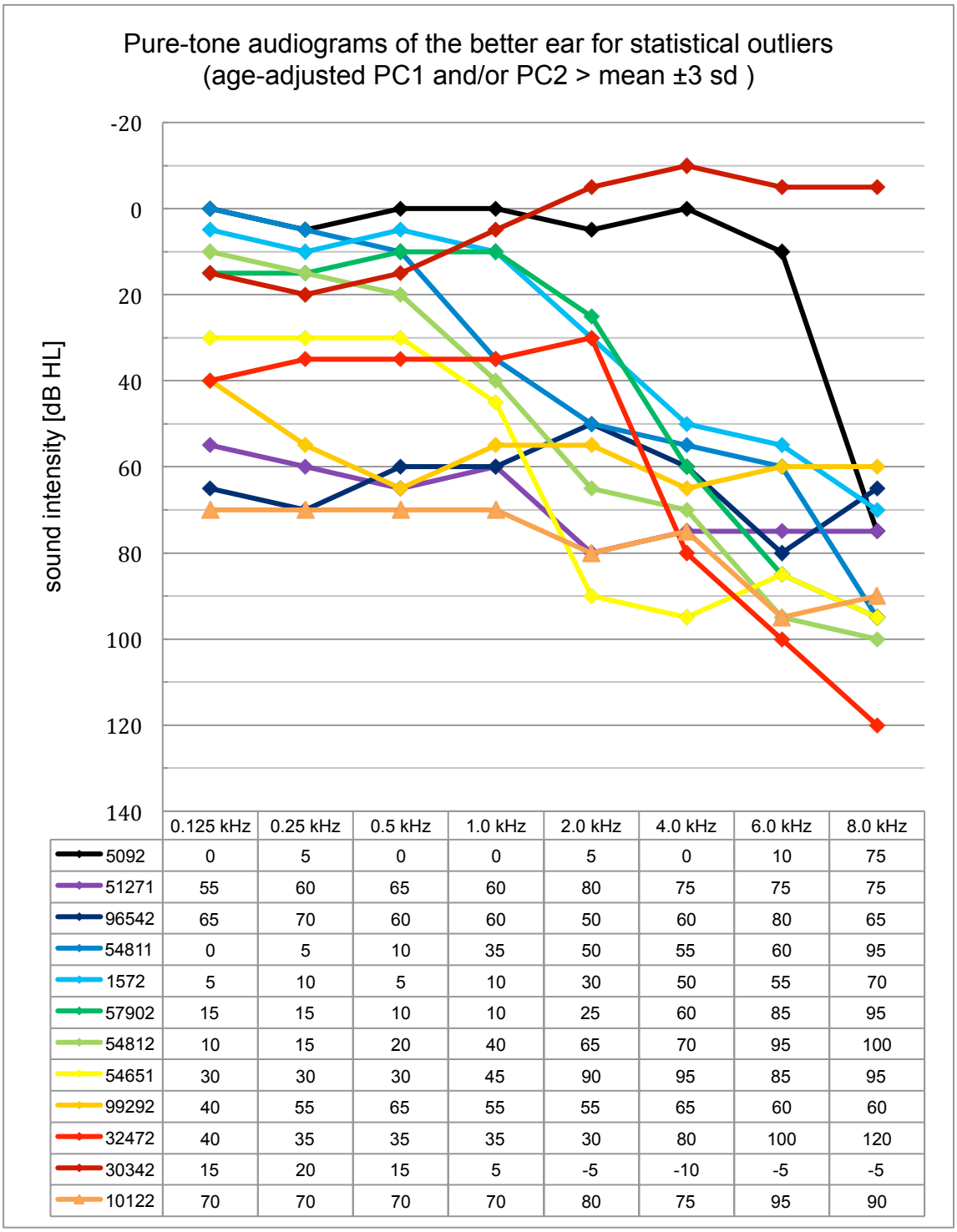


Figure 10 A high PC2 value indicates high frequency HL

Subjects were divided into three groups according to their age-adjusted PC2 values (low, medium and high). For each group average PTTs per frequency were obtained and plotted as average pure-tone audiogram per group. Error bars represent the standard error of the mean PTTs.

PC1 and PC2 outlier exclusion

12 outliers (age-adjusted PC1 and /or PC2> mean±3 SD) were determined in the current dataset. The pure-tone audiogram for the better ear was plotted for each of the 12 subjects (Figure 11) and examined for structures characteristic for early-onset HL, low frequency HL or noise trauma. After due consideration, it was decided to exclude individuals 51271, 96542, 54651, 99292, 32472 and 10122. All 6 individuals showed extremely raised thresholds both for the lower and higher frequencies with some of them exceeding thresholds for moderate HL [12]. Pure-tone audiograms for these individuals are characteristic for early onset HL rather than ARHI. Exclusion of cases with hearing pathologies other than ARHI aims to reduce variance in the dataset. After exclusion of these 6 outliers, 1303 individuals remained for analysis.



	0.125 kHz	0.25 kHz	0.5 kHz	1.0 kHz	2.0 kHz	4.0 kHz	6.0 kHz	8.0 kHz
5092	0	5	0	0	5	0	10	75
51271	55	60	65	60	80	75	75	75
96542	65	70	60	60	50	60	80	65
54811	0	5	10	35	50	55	60	95
1572	5	10	5	10	30	50	55	70
57902	15	15	10	10	25	60	85	95
54812	10	15	20	40	65	70	95	100
54651	30	30	30	45	90	95	85	95
99292	40	55	65	55	55	65	60	60
32472	40	35	35	35	30	80	100	120
30342	15	20	15	5	-5	-10	-5	-5
10122	70	70	70	70	80	75	95	90

Figure 11 Pure-tone audiograms for the better ear of statistical outliers

Pure-tone audiograms are displayed for the better ear of subjects whose PC1 or PC2 value deviated more than 3 standard deviations from the population mean. Exact pure-tone thresholds at each frequency measured for the better ear are shown in the table below the audiogram.

Description of study participants

The final dataset included 568 monozygotic (MZ) twins (MZ pairs: n=284), 630 dizygotic (DZ) twins (DZ pairs: n=315) and 105 twins without their twin sister, referred

to as unpaired twins (Table 9). Each of these three groups was characterized by their demographic characteristics (sample size (n), gender, mean age and age range) as well as by various measures of hearing ability (PTA, BEHL, age-adjusted PC1 and PC2) (Table 9). By ANOVA, there were no significant differences between MZs, DZs, and unpaired twins (Age: p= 0.8847; PTA: p=0.9125; BEHL: p= 0.8154; age-adjusted PC1: p=0.9743; age-adjusted PC2: p=0.1997). Means and variances showed no significant differences between MZs and DZs, which proves this twin sample suitable for future comparison in heritability studies.

Table 9 Demographic and hearing characteristics for different zygosity groups in TwinsUK

zygosity	n	gender (% female)	age		PTA (mean ± SD)	BEHL (mean ± SD)	age-adjusted PC1 (mean ± SD)	age-adjusted PC2 (mean ± SD)
			mean ± SD	range				
MZ	568	100%	61.57 ±8.79	40-83	22.2091 ±9.8217	18.5383 ±9.5208	-0.0715 ±1.8746	-0.0201 ±1.3695
DZ	630	100%	61.66 ±8.16	41-86	22.3512 ±9.8425	18.8829 ±9.3777	-0.0506 ±1.8255	0.0618 ±1.3418
unpaired twins	105	100%	62.01 ±8.73	41-83	22.6369 ±11.0424	18.8452 ±10.3422	-0.0370 ±2.2312	-0.1789 ±1.3783
total	1303	100%	61.64 ±8.48	40-86	22.3123 ±9.9279	18.7297 ±9.5148	-0.0586 ±1.8809	0.0067 ±1.3575

Samples from the TwinsUK ARHI study were divided into monozygotic (MZ) and dizygotic (DZ) twins and unpaired twins. Each group was described by their sample size (n), gender distribution (m=male, f=female), age (expressed as mean and standard deviation (SD) and age range). Pure-tone averages (PTAs), better ear hearing level thresholds (BEHLs) and age-adjusted principal components (PCs) are listed as mean and SD for each group.

Frequency of hearing loss in the TwinsUK cohort

To determine the incidence of HL in the TwinsUK cohort, thresholds were adapted from that published by the WHO [12], with slight HL defined as a PTA≥25 dB HL and moderate HL as PTA≥ 40 dB HL in this cohort. The histogram of PTAs for the better ear (Figure 12) shows the distribution of hearing ability in the TwinsUK cohort. The thresholds defined by the WHO were added as reference lines to the histogram. 66.39% of participants showed good hearing ability (no HL), while 27.55% and 6.06%

of subjects had slight or moderate HL, respectively. The group of participants with moderate HL also included the oldest participants (age range 54-86 years).

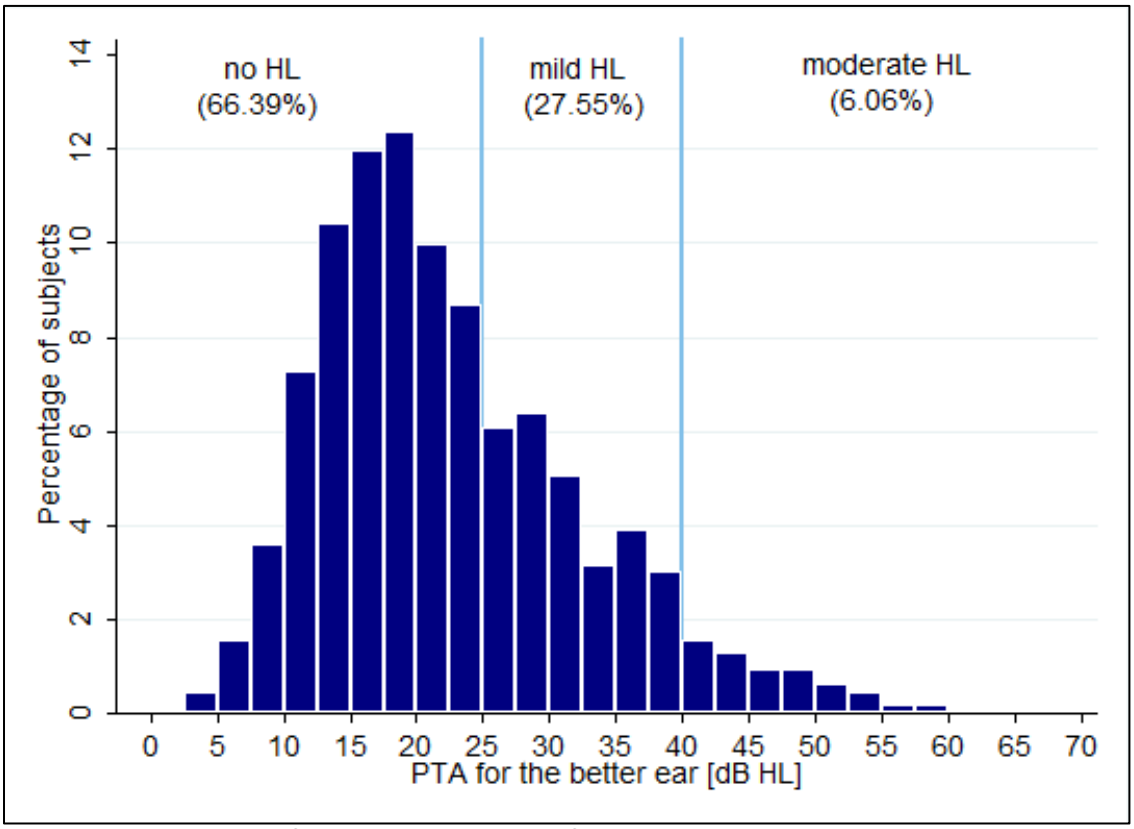


Figure 12 Histogram of pure-tone averages for the better ear

Figure 7 shows the distribution of pure-tone averages (PTAs) in the TwinsUK sample. 66.39% of subjects had good hearing ability, whereas 27.55% presented with slight and 6.06% of subjects with moderate hearing loss (HL).

Sensitivity and specificity of self-reported hearing loss

To determine the use of self-reported hearing in studies of ARHI, hearing test results were compared to self-reported HL. In total, 1228 participants answered the question concerning whether they had a hearing problem with 347 volunteers reporting hearing difficulties. 409 individuals were diagnosed with mild HL and 75 with moderate HL according to the hearing test. We used 2x2 contingency tables (Table 10 and Table 11) to calculate sensitivity and specificity for self-reported HL.

Table 10 Comparison of self-reported HL to slight HL as measured by pure-tone audiometry

self-reported hearing	Pure-tone audiogram results (PTA≥25 dB HL)		
	no HL	mild HL	total
no HL	687 (TN)	194 (FN)	881
HL	132 (FP)	215 (TP)	347
total	819	409	1228

The prevalence of mild hearing impairment (pure-tone average (PTA)≥ 25 dB HL) was compared to self-reported hearing difficulties.

Sensitivity= 215/(194+215)=0.53

Specificity=687/(687+132)=0.84

Self reported hearing in comparison to slight HL diagnosed using the PTA showed 52.6% sensitivity to detect true HL and showed 83.9% specificity to correctly identify healthy hearing individuals.

Table 11 Comparison of self-reported HL to moderate HL as measured by pure-tone audiometry

self-reported hearing	Pure-tone audiogram results (PTA≥40 dB HL)		
	no HL	moderate HL	total
no HL	871 (TN)	10 (FN)	881
HL	282 (FP)	65 (TP)	347
total	1153	75	1228

The prevalence of moderate hearing impairment (pure-tone average (PTA)≥ 40 dB HL) was compared to self-reported hearing difficulties.

Sensitivity= 65/(65+10)=0.87

Specificity= 871/(871+282)= 0.76

Self reported hearing in comparison to moderate HL diagnosed using the audiogram showed 86.7% sensitivity to detect true HL and showed 75.5% specificity to correctly identify healthy hearing individuals.

Association of hearing ability with environmental risk factors

Age-adjusted PC1 and PC2 values were compared between exposed and unexposed individuals for all environmental risk factors covered in the questionnaire. Response

rate was reduced for specific risk factors (i.e. 86% for exposure to otitis media during childhood) due to slight changes in the questionnaire throughout the study. Prevalence of risk factor exposure and age-adjusted PC1 and PC2 values for both exposed and unexposed volunteers are presented in tables and box-plots in the Appendix chapter 2.2- 2.13.

Student's t-test was conducted to determine whether groups of exposed and unexposed individuals showed significantly different PC1 and PC2 scores. The results of the t-tests (p-value) are shown for all binary risk factors in supplementary data 2-12. Individuals exposed to otitis media in adulthood (p=0.0041), chronic otitis media (p=0.0499) or eardrum operations (p=0.0019) showed significantly higher PC1 scores than unexposed individuals. Significantly higher PC2 values were determined for subjects exposed to explosions (p=0.0200) compared to unexposed participants. The individuals with self-reported hearing difficulties showed significantly different mean PC1 and PC2 (p<0.0000) values compared to the ones with self-reported normal hearing. No significant difference could be detected in hearing ability for subjects of different occupation or with different levels of occupational noise exposure.

In the factor analysis, factor 1 took mainly exposure to otitis media and eardrum operations into account, supporting the hypothesis that these exposures might not be independent of each other.

In the stepwise regression, only exposure to otitis media during childhood was significantly associated with age-adjusted PC1 (beta± SE=1.13±0.49; p=0.023; 95%CI: 0.16-2.11), however, this association accounted only for 2% of the variance in PC1 values (R^2=0.02). PC2 was significantly associated with exposure to noisy handiwork (beta± SE=0.82± 0.23, p=0.001; 95% CI: 0.36-1.28) and occupational noise exposure for less than 1 year (beta± SE=0.74±0.37, p=0.050; 95% CI: $5.50x10^{-4}$-1.47); nevertheless, both predictor variables together only explained 3% of the variance in PC2 values (R^2=0.03).

Reproducibility of the pure-tone audiogram

To examine the reproducibility of the pure-tone audiogram, hearing test results were compared within individuals that repeated the pure-tone audiogram at different visit dates. 117 individuals completed the audiogram at least twice. Subjects included in this analysis had a mean age of 62.30 years (±7.61 SD). There was an average gap of 16.46 months (±6.17 SD) between both study visits, ranging from 6 to 30 months. The

PTA calculated for the better was compared between both visits within the same subject.

Repeated hearing measurements showed a strong positive correlation (r=0.91). The Bland Altman plot [15] depicts the difference between repeated PTA measures against the mean of corresponding pairs of repeated measures (Figure 13). The mean difference between the PTA for the first visit compared to a later visit was PTA(Δ)=-2.082 dB HL (95% CI: -2.762 to -1.402), indicating that hearing deteriorated slightly by the later visit. The majority of mean PTAs between repeated hearing tests clustered in the lower PTA range (average PTA< 30 dB HL).

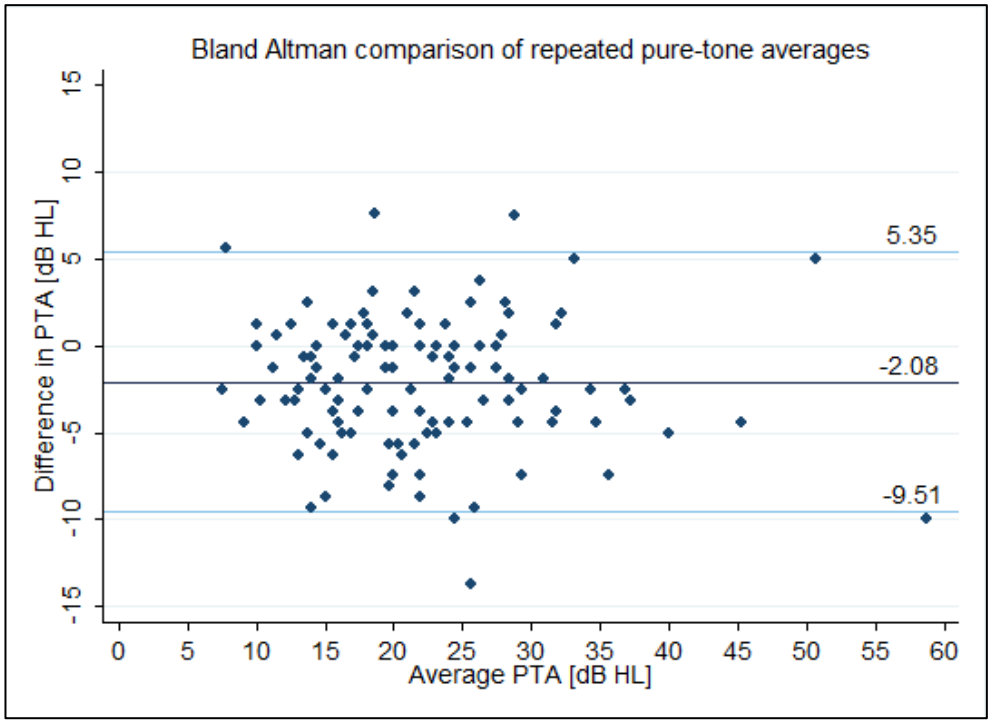


Figure 13 Bland Altman Plot for measurement of repeatability of pure-tone audiometry

The average PTA between both tests ranged from 7.50 dB HL to 58.75 dB HL. Pitman's test of difference in variance between both measurement groups was not significant (p=0.46). The mean difference between both tests was -2.08 dB HL (navy horizontal line). This corresponded with increased PTA measured at the second visit, indicating worse hearing. Light blue lines: limits of agreement (reference range for difference).

Discussion

ARHI is known to be a very heterogeneous trait, determined by environmental [16,17,18], genetic [9,19,20,21,22] and possibly epigenetic factors [23,24]. Pure-tone audiometric measures of hearing impairment in ageing cohorts has shown that ARHI is first detectable as an increase in PTTs for the higher frequencies moving gradually to

the lower frequencies, causing a characteristic downslope in the audiogram [25]. Standard pure-tone audiometry measures the lowest sound intensity an individual can hear at different frequencies within the human hearing frequency spectrum [1]. Assuming that hearing is measured for both ears separately at various frequencies, a multitude of variables per patient can be expected. To facilitate interpretation of hearing datasets, summary methods have been applied.

To determine the most suitable method to summarise hearing ability as measured by pure-tone audiometry, commonly used summary methods (PTA, BEHL, PTAs for different frequency ranges, and PCA) were applied to the audiograms measured in TwinsUK. It was decided to calculate all measures from thresholds for the better ear, as it is considered to be less representative of environmental factors affecting hearing ability. Exposure to environmental factors that affect primarily one ear would not represent natural age-related hearing and therefore bias the search for genetic factors. Furthermore, differences between the PTA calculated for both ears were limited ($m(\Delta)<$ 5 dB HL). In general, HL increased for the higher frequencies (4.0-8.0 kHz) and was lowest in the mid-frequencies (1.0-2.0 kHz) (Table 4). The trend in increasing HL for the higher frequencies was further reflected in an increasing correlation with age for the higher frequencies (Table 4). Furthermore, HL showed the highest variance at the higher frequencies (Table 4, $PTT_{2.0}$-$PTT_{8.0}$) indicating that hearing ability is most heterogeneous at this frequency range. This data shows that older subjects in TwinsUK show the typical high frequency HL and therefore sloping audiogram as described by *Schuknecht and Gacek* [5]. It thus proves this sample suitable for study of hearing ability with age.

In addition to averaging methods, a PCA was conducted. This method was first reported in hearing research by *Huyghe et al.* [9], where the first three PCs reflected 87.7% of the variance in PTTs and represented the magnitude, slope and concavity of the audiogram, respectively [9]. In TwinsUK only the first two PCs showed an eigenvalue≥ 1 (PC1: 3.78-4.34; PC2: 1.74-1.85) (Table 5, Table 7). The eigenvalue for PC3 (eigenvalue=0.86-0.70) did not reach the threshold of 1 used to select important PCs in this study (data not shown). In total, PC1 and PC2 explained together 70.3% and 76.0% (Table 5, Table 7) of the variance in PTTs in the age-adjusted and unadjusted PCA, respectively. It was assumed that the percentage of variance explained was inflated due to age differences for the unadjusted PCA. In the unadjusted analysis age, a known risk factor for ARHI, was not adjusted for. In the age-adjusted PCA, PCs were calculated from age-adjusted PTTs. Due to the increasing

correlation with age for the higher frequencies, it was thought more appropriate to adjust each threshold separately for age rather than adjusting the resulting components for age. Furthermore, both the adjusted and unadjusted PCA were calculated from log-transformed PTTs to respect the assumption of normal distribution.

In both PCAs, PC1 gave a measure of the horizontal threshold shift in the audiogram very similar to the "magnitude" described by *Huyghe et al.* [9], while PC2 represented the slope of the audiogram. PC1 increased with increasing HL over all frequencies and was highly correlated with the PTA (r=0.80). PC2 was positively correlated with the slope of the audiogram; a high age-adjusted PC2 value indicated a steep downslope for the higher frequencies, while a low age-adjusted PC2 value represented similar hearing ability for the lower and higher frequencies (a "flat" audiogram).

Measures like the PTA or BEHL represent averages over all or selected frequency ranges, respectively, thereby neglecting important information in the shape of the audiogram. In contrast, PCs reflected two important structures of the audiogram, the threshold shift overall frequencies and the slope of the audiogram. While PC1 appears to be very similar (and highly correlated) to the average measures (PTA or BEHL), PC2 represents an additional structure neglected by the PTA and the BEHL. According to Schuknecht et al. [5] sensory ARHI affects the higher frequencies first, creating a downslope in the audiogram for these frequencies. This typical downslope with increasing age was shown for audiograms of TwinsUK samples (Table 4). Therefore, a method detecting the shape of the audiogram would be of particular importance in case of ARHI.

Z-scores provide a standard tool used in many phenotypes to compare phenotypic measures of an individual to that of an appropriate reference population. In ARHI research, Z-scores have been applied particularly to measure HL in the older population, by calculating the difference in standard deviations from normal hearing ability for individuals of the same gender and age-range [8,26]. While this method measures the shape of the audiogram, reference values are not available for older elderly (>70 years old). It remains to be explained why the reference values for expected hearing ability with age, only provide measures for individuals below the age of 70. With an increasing live expectancy in developed countries [27], it would be particularly interesting to measure hearing ability in the age range of 40-90 years.

In conclusion, PCA was concluded to be the most appropriate method to reflect hearing ability in an ageing population as it takes both the threshold shift and the shape of the audiogram into account, while other methods either neglected the shape or excluded the oldest, and likely most affected, participants.

Pure-tone audiograms with extreme PC1 and/or PC2 scores deviating more than 3 standard deviations from the population mean were examined in more detail. Assuming a normally distributed trait, 99.73% of PC1 and PC2 values would be expected to fall into the range of 3 standard deviations around the mean [28]. This criterion was used to identify subjects with audiograms deviating from the majority of the sample and warranted further inspection of the pure-tone audiograms for the respective 12 individuals. It was decided to exclude 6 of the 12 individuals due to suspected early onset HL. All of these individuals showed raised PTTs (PTT≥30 dB HL) over all frequencies, which did not conform to our definition of ARHI affecting primarily the higher frequencies.

It was decided to study purely female participants out of two reasons. First, the TwinsUK cohort includes more female participants than males, because it was originally created to study osteoarthritis and osteoporosis in women [29,30,31], in addition women are more likely to volunteer for medical studies than men. Secondly, ARHI has been shown to be more pronounced in men than women [32] and due to this gender difference, many previous studies focused primarily on men or mixed gender cohorts [16,17,20,21]. Accordingly, the aim of this study was to explore factors causing ARHI particularly in ageing women. The age range of this study was slightly broader than for other hearing cohorts [7,16,33], mainly influenced by the minimum recruitment age, which was lower than for most other ARHI cohorts [7,16,17,33]. However, some individuals in the Beaver Dam offspring study [18] were even younger. Consequently, the chosen age range was comparable to the age range of other ARHI cohorts [7,17,34]. The low recruitment age was decided for in respect of all ageing phenotypes studied as part of the Healthy ageing twin study, which included the hearing data collection.

In accordance with grades of hearing impairment defined by the WHO [12], 33.60% of TwinsUK hearing subjects were defined as having HL (27.55% and 6.06% of volunteers were affected by mild and moderate HL, respectively). In the Beaver Dam hearing cohort (mean age: 65.8 years), a mild HL prevalence of 45.9% (PTA(0.5-4kHz)>25dB HL in the worse ear) was reported [17] and the prevalence of mild and

moderate HL in the Blue Mountain study (mean age: 67 years (95% CI: 66.7-67.4)) reached 39.1% and 13.4% [35]. In general, overall hearing ability was slightly better in TwinsUK (mean age: 61.64 years± 8.48 SD) than in comparable hearing cohorts, however the mean age of the TwinsUK hearing sample was below that of comparable cohorts.

Self-reported HL, a measure of ARHI used in previous studies [7,21] was covered by questionnaire (Appendix chapter 2.1). To determine the reliability of self-report in comparison to pure-tone audiometry, the sensitivity and specificity of self-reported HL against thresholds for mild and moderate HL were calculated. The sensitivity to determine mild HL was low (sensitivity=0.53) but increased for the more severe moderate HL (sensitivity=0.87). At the same time specificity to identify correctly individuals unaffected by mild HL (specificity=0.84) decreased for moderate HL (specificity=0.76). The sensitivity determined for self-reported HL in TwinsUK was lower than in the Blue Mountain study (mild HL: 0.78, moderate HL: 0.93)[35]. We assume that self-reported HL is sufficiently sensitive to determine moderate HL in a screening test. Nevertheless, these data support the use of a diagnostic test over self-reported hearing data.

The effect of environmental exposure on hearing ability was explored using information collected in the questionnaire (Appendix chapter 2.1). In the Welch's t-test individuals exposed to otitis media as an adult (p=0.0041) or chronic otitis media (p=0.0499) and eardrum operations (p=0.0019) showed significantly higher PC1 scores than volunteers unexposed to these factors. Participants exposed to explosions showed significantly higher PC2 scores (p=0.0200) than unexposed participants. We hypothesized that exposure to otitis media at different stages of development and eardrum operations might be dependent risk factors. Otitis media is more prevalent in individuals with insufficient drainage of the middle ear via the Eustachian tube and re-occurring infection of the middle ear cavity can result in pressure on - and ultimately rupture of - the tympanic membrane. Tympanostomy or insertion of a "grommet" into the eardrum was a common treatment for chronic otitis media but is now discredited [36]. Factor analysis was conducted to determine correlations between all risk factors. PC1 and PC2 were regressed against all risk factor exposures in a multivariate stepwise regression. In the factor analysis factor 1 was dominated by exposure to otitis media at different developmental stages and eardrum operations, thereby confirming our hypothesis. In addition, only exposure to otitis media as an adult and explosions remained significantly associated with PC1 and PC2 in the stepwise regression,

respectively. Nevertheless, both exposure to otitis media during adulthood and exposure to explosions explained only minor parts (1% and 0.62%) of variance in PC1 and PC2 scores, respectively. Noise exposure (both recreational and occupational) had no significant effect on hearing ability in TwinsUK, unlike reported for other samples (chapter 1, [16,17,18,37]). These results might be explained by the reduced noise exposure, increased personal hearing protection standards and younger age range of the presented sample. Furthermore, the purely female composition of this sample might have reduced the risk of occupational noise exposure (i.e. work in noisy factories).

To test whether the audiogram was reproducible, the test results of participants that completed the test more than once were compared using a Bland Altman comparison, a method designed to measure agreement between two different methods or repeated measures of the same trait. It is commonly assumed that a high correlation between two measurements also indicates a high agreement between measures. However, a correlation only describes a relationship between two variables (i.e. x increases with increasing values for y) and the magnitude of the correlation coefficient measures the strength of this relationship. A high correlation would be achieved if most paired values (i.e. x and y measured in the same individual) clustered along a straight line. Yet, a high correlation would not detect whether one measure was consistently higher or lower in all tested individuals. To measure true agreement between repeated measures, Bland and Altman examined graphically the difference in measurements against the average of both variables. Accordingly, the difference in PTA between test visit 1 and test visit 2 was calculated per subject and plotted versus the mean PTA between both tests. As hearing test circumstances were assumed to be constant for both visits, it would be wrong to assume that only one of both tests measured the true value of hearing, and therefore compared the second test to this allegedly true value. Thus, the average between both tests was assumed to be closest to the true value. In case of perfect reproducibility, measures would be expected to show no difference and therefore be equal to their average. Good reproducibility is indicated if repeated measures show a low difference between each other and the differences between measures for the same individuals cluster around 0. For the TwinsUK cohort, repeated measures showed a strong correlation (r=0.91). However, the difference between measures clustered around ΔPTA=-2.082 dB HL (95% CI: -2.762 to -1.402). This indicated that in 95% of the population, the PTA calculated from the second hearing test was around 2 dB HL higher than in the first test. This change in hearing ability over a mean period of 16.46 months (±6.17 SD) is to be expected in an ageing population. *Cruickshanks et al* [17] reported that the odds of HL increased by 1.88 per 5 years

increase in age (OR=1.88, 95% CI:1.80-1.97). In addition, a change of 2 dB HL in PTA appears very low compared to the PTA standard deviation from the mean (SD=10.3207) measured for all 1309 particpants (Table 4), ie only 0.2 SD. PTAs rather than PCs were chosen for the reproducibility analysis as the PTA values were better to interpret on a dB HL scale. It was therefore shown that the pure-tone audiogram used in this analysis showed high reproducibility.

There were limitations to this study. All hearing tests were conducted in a quiet room rather than in a sound isolated booth. A sound isolated booth would represent the perfect testing environment but was financially unfeasible for this study. To ensure that ambient noise levels remained within the recommended range [4], they were monitored on a regular basis. In addition, the hearing test was administered by a small group of research assistants including LW. All test personnel were trained to the same standards and training was repeated on a regular basis. Furthermore, the pure-tone audiogram was strictly automated to reduce tester-bias. Our records were not sufficient to test for an administrative effect on the hearing test results although this has been shown for other traits collected (ie heat pain threshold) it would not be expected in standardised pure-tone audiometry. In general, the volunteers recruited for this study were of better health and showed better hearing ability than reported for comparable hearing cohorts [17,35]. This bias towards better health might be caused by the recruitment and testing methods. Volunteers were invited to travel to St. Thomas hospital to conduct a test-battery of medical assessments. Home visits could not be offered due to lack of transport facilities for medical equipment and financial reasons. Thus, less healthy individuals were less likely to take part in this study. Nevertheless, previous studies in the TwinsUK cohort have shown that this cohort is representative of a singleton population [38]. Finally it should be acknowledged that using PCA as a measure of ARHI also shows limitations. PC loadings differ slightly in different study population (although the general structure remained) [9] and might therefore be more complicated to adapt than PTAs, which are measured in dB HL. In addition, interpreting of the scale of HL is more complicated for PC values than for summary methods measured in decibel.

References

1. Maltby MT (2002) Principles of hearing aid audiology: Whurr.
2. Graham J, Baguley D (2009) Ballantyne's Deafness: Wiley-Blackwell.
3. BSI (2010) BS EN ISO 389-9:2009. Acoustics. Reference zero for the calibration of audiometric equipment. British Standards Institution.

4. BSA (2011) Recommended Procedure for Pure-tone air-conduction and bone-conduction threshold audiometry with and without masking. Reading: British Society of Audiology.

5. Schuknecht HF, Gacek MR (1993) Cochlear pathology in presbycusis. Ann Otol Rhinol Laryngol 102: 1-16.

6. (2000) A Test for the Diagnosis of Dead Regions in the Cochlea. British Journal of Audiology 34: 205-224.

7. Viljanen A, Era P, Kaprio J, Pyykkö I, Koskenvuo M, et al. (2007) Genetic and Environmental Influences on Hearing in Older Women. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences 62: 447-452.

8. Fransen E, Van Laer L, Lemkens N, Caethoven G, Flothmann K, et al. (2004) A novel Z-score-based method to analyze candidate genes for age-related hearing impairment. Ear Hear 25: 133-141.

9. Huyghe JR, Van Laer L, Hendrickx JJ, Fransen E, Demeester K, et al. (2008) Genome-wide SNP-based linkage scan identifies a locus on 8q24 for an age-related hearing impairment trait. Am J Hum Genet 83: 401-407.

10. Pyykkö IV, Toppila EM, Starck JP, Juhola M, Auramo Y (2000) Database for a hearing conservation program. Scandinavian audiology 29: 52-58.

11. Smith LI (2002) A tutorial on Principal Components Analysis.

12. World Health Organisation (2011) Grades of hearing impairment. In: Organization WH, editor. Prevention of Blindness and Deafness Geneva: World Health Organization.

13. Akobeng AK (2007) Understanding diagnostic tests 1: sensitivity, specificity and predictive values. Acta Pædiatrica 96: 338-341.

14. Welch BL (1947) The Generalization of `Student's' Problem when Several Different Population Variances are Involved. Biometrika 34: 28-35.

15. Martin Bland J, Altman D (1986) Statistical methods for assessing agreement between two methods of clinical measurement. The Lancet 327: 307-310.

16. Fransen E, Topsakal V, Hendrickx JJ, Van Laer L, Huyghe JR, et al. (2008) Occupational noise, smoking, and a high body mass index are risk factors for age-related hearing impairment and moderate alcohol consumption is protective: a European population-based multicenter study. JARO-Journal of the Association for Research in Otolaryngology 9: 264-276.

17. Cruickshanks KJ, Wiley TL, Tweed TS, Klein BE, Klein R, et al. (1998) Prevalence of hearing loss in older adults in Beaver Dam, Wisconsin. The Epidemiology of Hearing Loss Study. Am J Epidemiol 148: 879-886.

18. Nash SD, Cruickshanks KJ, Klein R, Klein BEK, Nieto FJ, et al. (2011) The prevalence of hearing impairment and associated risk factors: the Beaver Dam Offspring Study. Archives of Otolaryngology—Head & Neck Surgery 137: 432.

19. Unal M, Tamer L, Dogruer ZN, Yildirim H, Vayisoglu Y, et al. (2005) N-acetyltransferase 2 gene polymorphism and presbycusis. Laryngoscope 115: 2238-2241.

20. Friedman RA, Van Laer L, Huentelman MJ, Sheth SS, Van Eyken E, et al. (2009) GRM7 variants confer susceptibility to age-related hearing impairment. Hum Mol Genet 18: 785-796.

21. Garringer HJ, Pankratz ND, Nichols WC, Reed T (2006) Hearing Impairment Susceptibility in Elderly Men and the DFNA18 Locus. Arch Otolaryngol Head Neck Surg 132: 506-510.

22. Nolan LS, Maier H, Hermans-Borgmeyer I, Girotto G, Ecob R, et al. (2013) Estrogen-related receptor gamma and hearing function: evidence of a role in humans and mice. Neurobiology of Aging 34: 2077.e2071-2077.e2079.

23. Provenzano MJ, Domann FE (2007) A role for epigenetics in hearing: Establishment and maintenance of auditory specific gene expression patterns. Hearing Research 233: 1-13.

24. Friedman L, Avraham K (2009) MicroRNAs and epigenetic regulation in the mammalian inner ear: implications for deafness. Mammalian Genome 20: 581-603.

25. Gates GA, Mills JH (2005) Presbycusis. The Lancet 366: 1111-1120.

26. World Health Organisation (2009) United Kingdom: general health statistical profile. WHO.

27. Office for National Statistics (2009) National Projections 2009: UK population to exceed 65m by 2018. Office for National Statistics.

28. Pukelsheim F (1994) The Three Sigma Rule. The American Statistician 48: 88-91.

29. Moayyeri A, Hammond CJ, Valdes AM, Spector TD (2012) Cohort Profile: TwinsUK and Healthy Ageing Twin Study. International Journal of Epidemiology.

30. Spector TD, MacGregor AJ (2002) The St. Thomas' UK Adult Twin Registry. Twin Research 5: 440-443.

31. Spector TD, Williams FM (2006) The UK adult twin registry (TwinsUK). Twin Research and Human Genetics 9: 899-906.

32. Gates GA, Couropmitree NN, Myers RH (1999) Genetic associations in age-related hearing thresholds. Arch Otolaryngol Head Neck Surg 125: 654-659.

33. Gates GA, Cooper Jr J, Kannel WB, Miller NJ (1990) Hearing in the elderly: the Framingham cohort, 1983-1985. Part I. Basic audiometric test results. Ear and Hearing 11: 247.

34. Chia EM, Wang JJ, Rochtchina E, Cumming RR, Newall P, et al. (2007) Hearing impairment and health-related quality of life: the Blue Mountains Hearing Study. Ear and Hearing 28: 187-195.

35. Sindhusake D, Mitchell P, Smith W, Golding M, Newall P, et al. (2001) Validation of self-reported hearing loss. The Blue Mountains Hearing Study. International Journal of Epidemiology 30: 1371-1378.

36. Lous J, Burton M, Felding J, Ovesen T, Rovers M, et al. (2005) Grommets (ventilation tubes) for hearing loss associated with otitis media with effusion in children. Cochrane Database Syst Rev 1.

37. Dalton DS, Cruickshanks KJ, Wiley TL, Klein BEK, Klein R, et al. (2001) Association of Leisure-Time Noise Exposure and Hearing Loss: Asociación entre exposición a ruido durante el tiempo libre e hipoacusia. International Journal of Audiology 40: 1-9.

38. Andrew T, Hart DJ, Snieder H, de Lange M, Spector TD, et al. (2001) Are twins and singletons comparable? A study of disease-related and lifestyle characteristics in adult women. Twin Res 4: 464-477.

Chapter 3: The classical twin model and heritability of ARHI in TwinsUK

Abstract

ARHI is a complex trait caused by both genetic and environmental factors, however, the proportion of variance in hearing ability explained by genetic or environmental factors is population specific. The aim of this chapter was to determine the relative influence of both additive genetic and environmental factors on the variance in hearing ability in older subjects from the TwinsUK cohort.

Heritability is defined as the variance in a trait explained by genetic factors. To decompose the variance seen in a trait into its causal components, twin studies have long been applied. Monozygotic twin pairs were assumed to share all of their genetic material, while dizygotic twin pairs share on average half of their alleles, like normal siblings. Both monozygotic and dizygotic twin pairs are exposed to an increased proportion of shared environment, if raised together. Comparison of trait covariance within monozygotic and dizygotic twin pairs can thus be used to partition trait variance into genetic and environmental causal factors. In this study the heritability of hearing ability (PC1, PC2) was estimated based on two methods: Falconer's formula and structural equation modelling founded on the classical twin model. Resulting estimates were compared between the two methods and the effect of age on heritability estimates explored.

Moderate heritability estimates were determined for both age-adjusted PC1 (h^2= 61% (95% CI: 54-67)) and PC2 (h^2=56% (95% CI: 49-63)). Age-adjustment of PC1 and PC2 values decreased the proportion of trait variance explained by environmental factors shared within twin pairs. In general, estimates achieved by Falconer's formula and structural equation modelling were very consistent. A model including additive genetic factors and environmental factors not shared within twin pairs gave the highest likelihood under the observed phenotypic variance in PC1 and PC2. In the analysis stratified by age, heritability estimates for both PC1 and PC2 increased with age of the subjects from 57% -77% and 49% - 58%, respectively.

The moderate heritability estimated for hearing ability supports the hypothesis that ARHI is determined to a high proportion by individual genetic variation in TwinsUK. The

genetic variants underlying this moderate heritability merit further investigation in genetic association studies.

Introduction

Complex traits are by definition caused both by our genes and the environmental we are exposed to. The study of epidemiology aims to observe the occurrence of complex traits and determine the exact factors increasing the risk of developing this trait, while quantitative genetics aims to estimate the extent of genetic and environmental factors on a specific phenotype. The results of these heritability studies can give insight into the power of gene mapping studies and help to determine the focus of further experiments.

The variability observable for a trait if measured in the population is referred to as phenotypic variance (V_P) and can be determined by different factors, including additive (A) and non-additive (D) genetic factors, common environmental factors (C) and unique environmental factors (E). The proportion of variance due to each of these independent influences is expressed as variance components: V_A, V_D, V_C, V_E. The phenotypic variance is expressed as the sum of the specific variance components (Equation 7).

$$V_P = V_A + V_D + V_C + V_E$$

[Equation 7, components of phenotypic variance]

The phenotypic variance can be expressed as the sum of latent variance components (V_A, V_D, V_C, V_E) due to additive genetic effects (A), non-additive genetic factors (D), common(C) and unique (E) environmental factors [1].

Common environmental factors include all environmental exposures shared between individuals of the same population, whereas exposure to unique or unshared environmental factors applies only to single subjects of the population under study.

According to Equation 7, phenotypic variance due to genetic factors is defined by two variance components: V_A and V_D. Additive genetic factors (A) refer to the sum of effects of every allele in the genome influencing the trait. Dominance or non-additive genetic factors (D), represent the effect of alleles on the trait by allele interaction at the same (dominance) or different loci (epistasis). The heritability of a trait is defined as the proportion of variance in a trait determined by genetic factors. Broad sense heritability (H^2) refers to the proportion in variance due to all genetic components (V_A+V_D)

(Equation 8), whereas narrow sense (h^2) heritability refers to the proportion of phenotypic variance only due to additive genetic components (V_A) (Equation 9)[1].

$$H^2 = \frac{V_A + V_D}{V_P}$$

[Equation 8 Broad sense heritability]

Broad sense heritability (H^2) is defined as the proportion of phenotypic variance (V_P) due to additive (V_A) and non additive (V_D) genetic variance components.

$$h^2 = \frac{V_A}{V_P}$$

[Equation 9 Narrow sense heritability]

Narrow sense heritability (H^2) is defined as the proportion of phenotypic variance (V_P) due to additive (V_A) genetic variance components.

Broad sense heritability gives an estimate of the proportion of variance in a trait determined by genetic factors. Narrow sense heritability, on the other hand, is also referred to as breeding value, because it estimates the proportion of V_P determined by the alleles an individual inherits from its founders. Heritability is a population specific measure and can differ for the same trait between populations. This can be explained by genetic variability (i.e. differences in alleles and allele frequencies) and differences in environment between populations, which could influence a trait.

If a trait were primarily determined by genetic factors, genetically identical individuals would be assumed to have the same risk of developing this trait. Accordingly, raised phenotypic concordance would be expected in monozygotic (MZ) twin pairs, who are assumed to share all their genetic variations, compared to pairs of unrelated individuals. The probandwise concordance rate (PCR) (Equation 10), calculated as twice the number of concordant affected twin pairs over the number of affected individuals, gives the risk of the twin of an affected co-twin to be affected as well. Higher PCRs for MZ twin pairs compared to dizygotic (DZ) twin pairs indicate a genetic influence in the trait.

$$PCR = \frac{2x\ concordant\ affected\ twin\ pairs}{2x\ concordant\ affected\ twin\ pairs + discordant\ twin\ pairs}$$

[Equation 10, Probandwise concordance rate]

The same accounts for traits determined primarily by environmental factors. Siblings growing up in the same family environment, or even sharing the time in uterus, would be expected to show higher concordance in environmentally determined traits than subjects raised or living in different environments. The special potential of twins in quantitative genetics has first been recognized by Galton [2] and later been described in more detail by Siemens [3], a German dermatologist who correlated mole counts in twins.

Non-additive (or dominant) genetic and unique environmental factors are difficult to distinguish in twins raised together and can thus not be estimated together in one model. However, comparing the correlation between MZ and DZ twin pairs can help to decide which of both variance components (V_D or V_E) to include in the model [4]. In this study an effect due to unique environmental factors rather than non-additive genetic factors will be assumed.

The classical twin model assumes that MZ twin pairs share all of their additive genetic factors and common environmental factors. Trait discordance in MZ twins is therefore supposed to be caused by environmental exposure unique to one of both twin siblings. DZ twin pairs, in contrast, share on average half of their alleles and thus half of their additive genetic factors, but all common environmental factors. Consequently, DZ discordance can be attributed to the unshared half of their additive genetic components and due to unique environmental exposure. A path diagram depicting the relationship within MZ and DZ twin pairs (“The classical twin model”) on the basis of variance components is shown in Figure 1.

The classical twin model makes four assumptions concerning the study sample and trait it is applied to:

1. Minimal or no gene x environment interactions for the studied trait.
2. The extent of shared environment is equal for both MZ and DZ twin pairs.
3. The phenotypic variance (V_P) in twins is representative of the V_P in the population.
4. There is no selective assortment due to the trait.

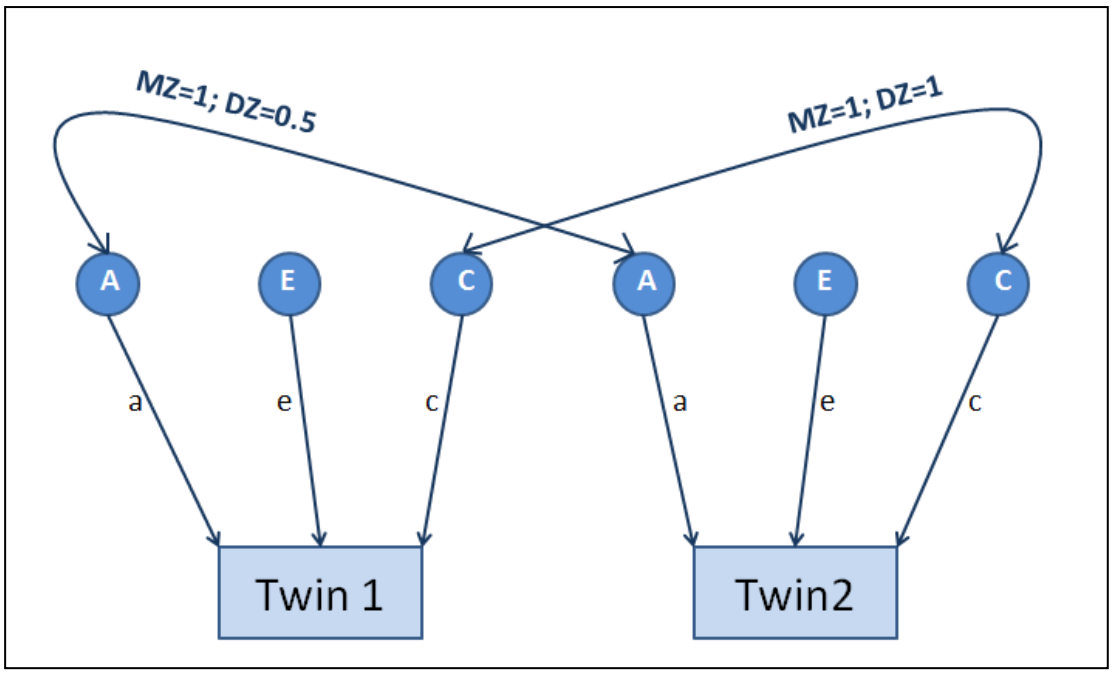


Figure 14 The classical twin model

The classical twin model describes the relationship in phenotype within twin pairs. The phenotype measured in both twins of a pair is indicated by blue boxes. Monozygotic twin pairs (MZ) share 100% (=1) of their additive genetic factors (A), while dizygotic twin pairs (DZ) share on average half of their alleles (=0.5; correlations are depicted as double headed arrows). Both MZ and DZ twin pairs are fully exposed to the same common environment (C). The single headed arrows represent path coefficients, measuring the effect of the latent variables on the phenotype. The phenotypic variance attributed to the specific variance components (A, E, C) can be calculated as the square of the respective path coefficients (ie $A=a^2$).

The described model (

Figure 14) allows the covariance within MZ (Cov_{MZ}) and DZ (Cov_{DZ}) twin pairs to be expressed as a function of the variance components and path coefficients.

$$Cov_{MZ} = A + C = a^2 + c^2$$

[Equation 11 Covariance within MZ twin pairs]

Covariance within MZ twin pairs can be expressed as sum of additive genetic (A) and common environmental (C) factors.

$$Cov_{DZ} = \frac{1}{2}\,A + C = \frac{1}{2}\,a^2 + c^2$$

[Equation 12 Covariance within DZ twin pairs]

Covariance with DZ twin pairs can be expressed as sum of half of the additive genetic (A) and all common environmental (C) factors.

Accordingly, heritability (H^2) can be estimated as twice the difference between MZ and DZ twin pair covariance. The within twin pair covariance is calculated using the intra-class correlation coefficient (ICC). This relationship was first described by Falconer [1] and is therefore referred to as Falconer's formula.

$$H^2 = a^2 = A = 2\times (ICC_{MZ} - ICC_{DZ})$$

[Equation 13, Falconer's formula]

Heritability (H^2) can be estimated as twice the difference in intra-class correlation coefficients (ICC) within MZ and DZ twin pairs.

Accordingly, estimates for C and E can be achieved as

$$c^2 = C = ICC_{MZ} - H^2 \quad \text{and} \quad e^2 = E = 1 - H^2 + c^2$$

To facilitate heritability studies on larger datasets, the above described formulas have been applied in structural equation modelling (SEM) software, like Mx [5]. Estimates for the latent variance components (A, C and E) are determined based on maximum likelihood estimation. SEM allows more complex models, including covariates as sex and age, to be fitted to the observed twin data and can give an estimate of the model fit.

Heritability analysis can predict the statistical power of gene mapping studies, as a high heritability represents high correlation between phenotype and genotype. However, heritability makes no claim about the architecture of a trait, i.e. whether the trait is caused by few genetic variations with high effect sizes or many variations with each low effect size [6]. This introduction focused mainly on heritability estimation in twin samples, however, other study designs, including family data and adoption studies, can be applied to compare the aggregation of a trait in genetically related and unrelated individuals or individuals exposed to the same family environment although genetically unrelated.

The heritability of ARHI has been the focus of several studies [7,8,9,10,11]. *Karlsson et al* [7], studied the heritability of ARHI at different age-groups in male twin pairs. This study focused specifically on a high-frequency hearing loss phenotype with age and therefore only examined pure-tone averages calculated over 3-8 kHz combined for both ears. He determined an increase in variation in HL explained by unique environmental factors with advancing age.

Gates et al [9], compared the aggregation of hearing thresholds in genetically unrelated individuals (spouses) to genetically related subjects (siblings and parent-child pairs) collected as part of the Framingham heart study cohort. In this analyses hearing with age was divided into three different phenotypes: normal hearing, abrupt high frequency hearing loss (sensory presbycusis) and flat hearing loss with age (strial presbycusis). These three hearing phenotypes were combined to study the heritability of presbycusis (including all phenotypes), sensory presbycusis (including subjects with normal hearing and sensory ARHI) and strial presbycusis (including normal hearing individuals and cases with strial presbycusis). Furthermore, the analysis was performed separately for males and females and for hearing thresholds at the low (0.23-1 kHz), medium (0.5-2 kHz) and high (4-8 kHz) frequency ranges. In all analysis phenotypic aggregation of hearing thresholds was higher for genetically related individuals than for unrelated subjects. Heritability estimates ranged from 25-55% (Table 12) and were reported to be higher in female genetically related individuals, probably due to lower exposure to occupational noise in women [9].

Christensen et al [11] studied the heritability of self-reported reduced hearing in older same sex twin pairs from Denmark. They applied both probandwise concordance rates and SEM based on the classical twin model. Probandwise concordance rates were higher in MZs compared to DZ twin pairs (0.58 compared to 0.47), indicating the

influence of genetic factors on the trait variance. In the SEM, the AE model provided the best model fit, with A= 40% (95% CI: 19-52).

Viljanen et al [8] studied the heritability of ARHI in female twins. Hearing ability was measured as the better ear hearing threshold level (BEHL), the better ear speech recognition threshold level (BESRL), self rated hearing problems and hearing impairment measured as a BEHL≥ 21dB. Heritability estimates for the continuous hearing traits (BEHL and BESRL) were determined using SEM based on an ADE twin model, while probandwise concordance rates [4] were calculated for binary traits (self-rated hearing and hearing impairment). Of all measured traits in this study of female twins, the BEHL showed the highest heritability (h^2= 75% (95% CI: 67-81)).

Huyghe et al [10], estimated the heritability for principal components (PCs) calculated from pure-tone audiometric data in related individuals. Here, heritability was estimated as twice the intraclass correlation coefficient and ranged from 66.3% for PC1 to 27.2 % for PC2 [10]. Heritability estimates for ARHI determined in the presented studies [7,8,9,10,11] are summarised in Table 12.

Table 12 Summary of Heritability studies on ARHI

reference	study sample	age range (years)	phenotype	probandwise concordance	heritability
Karlsson et al, 1997 [7]	male MZ and DZ twin pairs	35-45	hearing in the high-tone ranges (pure-tone average calculated over 3, 4, 6 and 8 kHz) for both ears combined	-	100%
		46-55		-	95%
		56-65		-	58.4%
		≥65		-	47.4%
Gates et al, 1999 [9]	genetically unrelated and genetically related individuals	husbands: 70.9±10.0 wives: 69.5±6.7 brothers: 59.7±10.0 sisters: 60.1±10.6	age-adjusted pure-tone average	-	26-35%
			age-adjusted pure-tone average in individuals with sensory presbycusis or normal hearing	-	35-55%
			age-adjusted pure-tone average in individuals with strial presbycusis or normal hearing	-	25-42%
Christensen et al, 2001 [11]	male and female same gender MZ and DZ twin pairs	70-102	Self-reported age-related hearing loss	MZ: 0.58 (95%CI:0.52-0.64) DZ: 0.47 (95% CI: 0.42-0.52)	40% (95% CI: 19-52)
Viljanen et al, 2007 [8]	female MZ and DZ twin pairs	63-76	better ear hearing threshold level (BEHL)	-	75% (95% CI: 67-81)
			better ear speech recognition threshold level	-	66% (95% CI: 55-74)
			self rated problems in hearing	MZ: 0.65 (95% CI: 0.55-0.74) DZ: 0.59 (95% CI: 0.49-0.70)	-
			hearing impairment (BEHL≥ 21 dB)	MZ: 0.81 (95% CI: 0.72-0.89) DZ: 0.44 (95% CI: 0.30-0.58)	-
Huyghe et al, 2008 [10]	mixed gender sibships	49-76	PC1	-	66.3%
			PC2	-	27.2%
			PC3	-	37.5%

This table summarises the study design and results of previous heritability analyses of ARHI. Each study is further described by the sample studied (gender, twins or families), the chronological age range of the study sample (shown as age range or mean age ± SD) and the exact phenotype covered (phenotype). The resulting heritability estimates are given by the applied estimation method (probandwise concordance rates for binary traits and heritability estimates for continuous and binary traits).

The objective of this chapter was to determine the heritability of hearing ability with age in female twins from the TwinsUK register using Falconer's formula and SEM in Mx and further investigate the effect of increasing age on heritability of hearing ability.

Materials and Methods

Study sample

This analysis included all individuals remaining after quality control and outlier removal (n=1303) as described in chapter 2.

Interclass and intraclass correlations

To give an estimate of within twin pair correlations by zygosity, age-adjusted PC1 and PC2 values within twin pairs were plotted against each other and a linear regression line fitted to the data. Plots were created for MZ and DZ twin pairs separately.

The intraclass correlation (ICC) measures the proportion of variance within a group in comparison to the variance seen between various subgroups. This measure has been used to assess the agreement between methods tested on the same individual and to measure the resemblance within families. In this analysis the ICC was applied to measure the resemblance in hearing ability (PC1, PC2) within twin pairs in comparison to resemblance within the all samples. To estimate the ICC, the variance within a twin pair was divided by the total trait variance determined for all twins of the same zygosity. The ICC ranges from 0 to 1, with a low ICC value indicating high variance within twin pairs approaching the variance in the population. In contrast, a high ICC represents a low variance (and therefore high resemblance) within pairs in comparison to the variance in the population. In case of a genetically determined trait, MZ twin pairs are supposed to present with a very similar phenotype, whereas within DZ twin pairs more variability in phenotype is expected. To estimate the broad sense heritability of hearing ability for PC1 and PC2, twice the difference in ICCs for MZs and DZs was calculated (Equation 13, Falconer's formula)[1].

Structural equation modelling based on the classical twin model

The genetic and environmental relationships within twin pairs as described by the classical twin model were expressed in structural equations. These structural equation models were fitted to the observed variance in PC1 and PC2 and goodness of model fit determined by maximum likelihood estimation. The aim of maximum likelihood estimation is to determine parameters for A, C and E, which make the observed twin covariance most likely. The maximum likelihood was reported as twice the negative natural logarithm of the likelihood. Nested models with reduced numbers of latent components (CE, AE and E) were compared to the full (ACE) model based on a likelihood ratio test [12]. The likelihood ratio test determines whether the improvement in model fit due to an increase in latent variables is significant. In the ratio test a chi-square statistic is fitted to the difference in likelihood between the full (ACE) and the respective reduced model (CE, AE and E). The degrees of freedom (df) for the chi-square statistic are determined as the difference in df (Δdf) between the full and the reduced model. A p-value was calculated based on the chi-square statistic, which showed whether the reduced model was significantly less likely under the observed

phenotype. In case of an equally well fitting reduced model, the chi-square statistic was expected to be non-significant (p>0.05). Both models were compared based on the principle of parsimony, which states that a simpler model should be preferred if it can explain the observed data equally well as a more complicated model including more latent variables. Akaike's information criterion (AIC) was used to compare model fit in respect to difference in degrees of freedom between two models and favoured the less parsimonious model (indicated by a low AIC value) [13].The AIC for the comparison of a nested to a full model was calculated according to Equation 14.

$$AIC = -2\Delta df + \Delta(-2logL)$$

[Equation 14 Akaike's information criterion]

With ∆df representing the difference in degrees of freedom between both models and ∆-2logL indicating the difference in twice the negative logarithm of the maximum likelihood achieved for the respective model.

Heritability analysis for different age-groups

The study sample was divided into three age groups with similar sample sizes per group (n=415-448). Broad sense heritability was calculated for each age group by calculation of ICCs for MZs and DZs separately using unadjusted PC values. Narrow sense heritability estimates for each age group were obtained using SEM based on the classical twin model as described above using unadjusted PC values.

Results

Study sample

For the heritability analysis, all subjects remaining after quality control (as described in chapter 2) were taken forward for analysis. In general, there were slightly more DZ twins in this sample than MZ twins (Table 13), however, no significant difference in mean PC1 and PC2 values could be detected between MZs and DZs (ANOVA, chapter 2).

Table 13 Population characteristics of the ARHI heritability study sample

zygosity	n	gender (% female)	age		PC1 (mean±sd)	PC2 (mean±sd)	age-adjusted PC1 (mean±sd)	age-adjusted PC2 (mean±sd)
			(mean ±SD)	range				
MZ	568	100%	61.57 ±8.79	40-83	-0.0200 ±2.0169	-0.0046 ±1.3435	-0.0715 ±1.8745	-0.0201 ±1.3695
DZ	630	100%	61.66 ±8.16	41-86	0.0113 ±1.9711	-0.0856 ±1.2529	-0.0506 ±1.8255	0.0618 ±1.3418
unpaired twins	105	100%	62.01 ±8.73	41-83	0.0616 ±2.3664	0.1272 ±1.3681	-0.0370 ±2.2312	-0.1789 ±1.3783
total	1303	100%	61.64 ±8.48	40-86	0.0017 ±2.0239	-0.0332 ±1.3029	-0.0586 ±1.8809	0.0067 ±1.3575

This table summarizes the characteristics of the all subjects used in the heritability study of ARHI in TwinsUK. The sample is divided into three subsamples by zygosity of the participants. Each subsample is characterized by the number of subjects (n), gender, mean chronological age and age range at hearing test. Furthermore, the 4 phenotypes analysed are summarised for each group as mean ± standard deviation from the mean.

Heritability of PC1 and PC2

First, the correlation within MZ and DZ twin pairs for age-adjusted PC1 and PC2 values was graphically examined (Figure 15, Figure 16). Values for MZ twins clustered more closely around the regression line than DZ values (Figure 15, Figure 16). Furthermore, the slope for the regression line was steeper for MZs than for DZs.

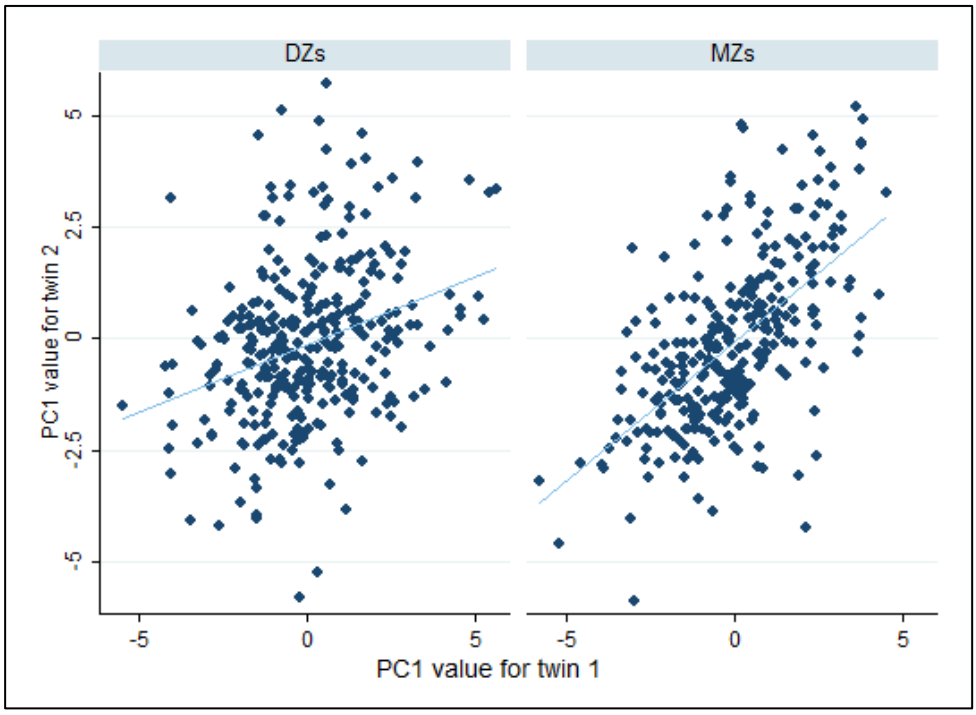


Figure 15 Scatter plots of age-adjusted PC1 values by zygosity

Age-adjusted PC1 values of one twin were plotted against the PC1 value obtained for their twin sibling. Two plots were generated, one for MZ twin pairs (right) and one for DZ twin pairs (left). A linear regression line (light blue line) was fitted to each scatter plot.

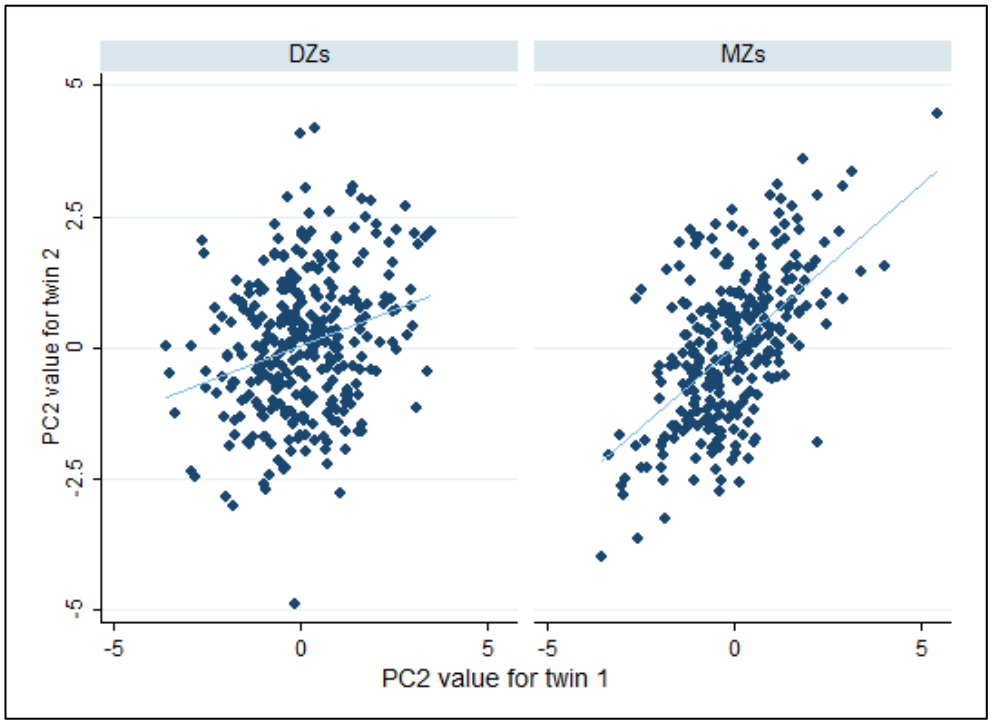


Figure 16 Scatter plots of age-adjusted PC2 values by zygosity

Age-adjusted PC2 values of one twin where plotted against the PC2 value obtained for their co-twin. Two plots were generated, one for MZ twin pairs (right) and one for DZ twin pairs (left). A linear regression line (light blue line) was fitted to each scatter plot.

The broad sense heritability was estimated using ICCs for MZs and DZs separately according to Falconer's formula (Equation 13). ICCs were higher for MZs than DZs for all measured phenotypes, as expected for a genetically influenced trait (Table 14). The heritability was higher for PC2 than for PC1 and estimates increased for age-adjusted phenotypes (Table 14). If variance in a trait was influenced by dominance genetic factors, ICC_{MZ} would be expected to be twice as high as ICC_{DZ} [4]. This was not the case for most of the phenotypes investigated here (Table 14). It was therefore decided to assume an ACE rather than an ADE model for further analyses.

Table 14 ICCs and broad sense heritability estimates for PC1 and PC2

Phenotype	ICC_{MZ}	ICC_{DZ}	H^2	A % (95% CI)
PC1	0.73	0.52	0.42	39 (23- 57)
age-adjusted PC1	0.62	0.33	0.58	61 (54- 67)
PC2	0.58	0.32	0.52	57 (50- 63)
age-adjusted PC2	0.57	0.27	0.74	56 (49- 63)

The intraclass correlation coefficients for MZ (ICC_{MZ}) and DZ (ICC_{DZ}) twin pairs were calculated for each phenotype and broad sense heritability (H^2) was calculated as twice the difference in ICCs for MZs and DZs ($H^2=2x(ICC_{MZ} - ICC_{DZ})$). For comparison, the percentage of phenotypic variance explained by additive genetics (A%) determined in SEM (Table 15) for the respective phenotype was added with 95% confidence intervals.

The results of the heritability analysis of hearing ability according to SEM are listed in Table 15. For all phenotypes (excluding unadjusted PC1 values), the reduced AE model fitted the data better than the full ACE model. This indicates that hearing ability with age is primarily determined by additive genic and unshared or unique environmental factors. For all phenotypes, narrow sense heritability ranged from 39%-61% and exceeded the percentage of variance explained due to unique environmental factors. The effect of common environmental factors in the ACE model was reduced after adjustment of PC1 and PC2 values for chronological age of subjects (Table 15). Narrow sense (SEM, Table 15) and broad sense heritability estimates (Falconer's formula, Table 14) were highly comparable.

Table 15 Results of the structural equation modelling used to estimate the influence of variance components (A, C, E) on hearing ability with age

phenotype	model fit			model comparison				univariate estimates % (95% CI)		
	model	-2LogL	df	Δ -2LogL	Δ df	χ^2 p-value	AIC	A	C	E
PC1 age-adjusted	ACE	-5181.81	1296	-	-	-	-	56 (33- 67)	4 (0-24)	39 (33- 47)
	AE	-5181.97	1297	0.17	1	0.68	-1.83	61 (54- 67)	-	39 (33-46)
PC1	ACE	-5229.91	1296	-	-	-	-	39 (23- 57)	33 (17- 48)	28 (23- 33)
	AE	-5243.28	1297	13.37	1	0.00	11.37	74 (69- 78)	-	26 (22- 31)
PC2 age-adjusted	ACE	-4355.78	1296	-	-	-	-	56 (34- 63)	0 (0- 19)	44 (37- 51)
	AE	-4355.78	1297	0.00	1	1.00	-2.00	56 (49- 63)	-	44 (37-51)
PC2	ACE	-4237.22	1296	-	-	-	-	44 (21- 62)	11 (0- 31)	44 (38-52)
	AE	-4238.31	1297	1.09	1	0.30	-0.91	57 (50- 63)	-	43 (37-50)

In the structural equation modelling, an ACE model (model) was fitted to the observed phenotypic variance in age-adjusted compared to unadjusted PC1 and PC2 values based on a maximum likelihood estimation (model fit, -2LogL). For each trait, three nested models were compared to the full ACE model, taking into account different causal factors: AE (additive genetics and unshared environmental factors), CE (shared and unshared environmental factors) and E (unshared environmental factors). Model fit for the full and respective reduced models were compared in a likelihood ratio test (Model comparison). Significance of the likelihood ratio test (χ^2 p-value) is determined in a chi-square statistic based on the difference in twice the negative logarithm of the likelihood (Δ-2LogL) and difference in degrees of freedom (Δdf) between the compared models. In addition, the Akaike's information criterion (AIC) gives a measure of parsimony of a reduced model in comparison to the full model. For each phenotype the ACE model fit and nested models with a better model fit (highlighted in grey) are shown. Model comparison is only given for nested models as they are compared to the full (ACE) model. Estimated variances explained by the specific causal factors (A= additive genetics, C= shared environment and E= unshared environment) are given with 95 % confidence intervals for each model.

Heritability analysis for different age groups

In this analysis unpaired twins were included as MZs or DZs, depending on their zygosity. By including unpaired twins in the heritability analysis, the variance in phenotype is increased, giving a more realistic heritability estimate for the population under study.

For the heritability analysis of different age groups, the sample was divided into three age groups with the aim to achieve similar sample sizes and an equal distribution of MZ and DZ twins per age group. Age groups 1 and 2 were of similar sample size, whereas group 3 included slightly less subjects (Table 16). The mean age between the groups differed by approximately 10 years (Table 16). The total study sample included more DZ twins than MZ twins, which was particularly reflected in age group 2, while the proportion of DZs to MZs was nearly 50% in the oldest group (age group 3, Table 16). As this analysis was aimed to detect an effect of age on the heritability of ARHI, unadjusted PC1 and PC2 values were used in this analysis.

Table 16 Characteristic of the ARHI sample divided into three age groups

age group	age range	mean age (SD)	n	n (DZ/MZ)	PC1(MZ) mean ±SD	PC1(DZ) mean ±SD	PC2(MZ) mean ±SD	PC2(DZ) mean ±SD
1	40-58	52.28 (4.52)	440	231/209	-1.1741 ±1.5704	-1.1254 ± 1.7256	0.4468 ±1.2670	0.3117 ±1.2087
2	59-65	62.14 (1.90)	448	248 /200	0.0284 ±1.8577	-0.0561 ±1.7652	-0.0805 ±1.2980	-0.1279 ±1.2190
3	66-86	71.04 (4.34)	415	206/209	1.2529 ±1.9420	1.2330 ±1.8101	-0.3488 ±1.3618	-0.4266 ±1.2521
Total	40-86	61.64 (8.48)	1303	685/ 618	0.0358 ±2.0527	-0.0290 ±1.9986	0.0071 ±1.3493	-0.0695 ±1.2595

This table summarizes the characteristics of the subjects used in the heritability analysis by age group. For each age group (1-3), chronological age range and mean age with standard deviation from the mean (SD) at hearing test are presented. The number of subjects per group (n) and proportion of MZ and DZ twins per group (n(MZ/DZ)) are listed. Furthermore, The mean unadjusted PC1 and PC2 values ± standard deviation from the mean (± SD) are given for MZ and DZ twin pairs, separately.

Heritability of PC1 and PC2 at different age ranges was estimated using both Falconer's formula (Table 17) as well as SEM (Table 18). Broad sense heritability for PC1 and PC2 increased with increasing age of the participants under study (H^2(PC1): 28%-74%; H^2(PC2): 44%-76%) (Table 17). This trend was confirmed by the results of the SEM used to estimate narrow sense heritability of PC1 and PC2 at different age groups (h^2(PC1): 57%-77%; h^2(PC2): 49%-58%)(Table 18). In general, the reduced

model considering additive genetic factors (A) and unique environmental factors (E) as variance components of hearing ability fitted the observed data best for both phenotypes (PC1, PC2) and all age groups. This data can give insight into the age range at which ARHI is most influenced by our genes, which is likely to be in the higher age groups. In comparison to each other, heritability estimates obtained by SEM were slightly increased compared to heritability estimates calculated using Falconer's formula (Table 17).

Table 17 Intraclass correlation coefficients and broad sense heritability for PC1 and PC2 at different age ranges

phenotype	age range	ICC_{MZ}	ICC_{DZ}	H^2	A % (95% CI)
PC1	40-58	0.52	0.38	0.28	57 (44- 68)
	59-65	0.60	0.38	0.44	60 (47- 69)
	66-86	0.78	0.41	0.74	77 (69- 83)
	Total	0.73	0.52	0.42	39 (23- 57)
PC2	40-58	0.50	0.28	0.44	49 (35- 61)
	59-65	0.54	0.34	0.40	54 (40- 64)
	66-86	0.62	0.24	0.76	58 (45- 69)
	Total	0.58	0.32	0.52	57 (50- 63)

The intraclass correlation coefficients for MZ (ICC_{MZ}) and DZ (ICC_{DZ}) twin pairs were calculated for each phenotype and broad sense heritability (H^2) was calculated as twice the difference in ICCs for MZs and DZs ($H^2=2x(ICC_{MZ} - ICC_{DZ})$). For comparison, the percentage of phenotypic variance explained by additive genetics (A%) determined in SEM (Table 18) for the respective phenotype was added with 95% confidence intervals.

Table 18 Results of structural equation modelling based on different age groups

phenotype	model Fit			model comparison				univariate estimates % (95% CI)		
	model	-2LogL	df	Δ -2LogL	Δ df	χ^2 p-value	AIC	A	C	E
PC1 age-group 1	ACE	-1638.51	432	-	-	-	-	39 (0- 67)	16 (0- 47)	45 (33- 60)
	AE	-1639.33	433	0.82	1	0.36	-1.18	57 (44- 68)	-	43 (32- 56)
PC1 age-group 2	ACE	-1747.43	442	-	-	-	-	39 (0.3- 68)	19 (0- 50)	42 (31- 56)
	AE	-1748.62	443	1.20	1	0.27	-0.80	60 (47- 69)	-	40 (31- 53)
PC1 age-group 3	ACE	-1597.99	408	-	-	-	-	72 (39- 83)	5 (0- 36)	23 (17- 32)
	AE	-1598.07	409	0.08	1	0.78	-1.92	77 (69- 83)	-	23 (17- 31)
PC2 age-group 1	ACE	-1393.32	432	-	-	-	-	40 (0- 61)	8 (0- 43)	52 (39- 67)
	AE	-1393.49	433	0.17	1	0.68	-1.83	49 (35- 61)	-	51 (39- 65)
PC2 age-group 2	ACE	-1430.96	442	-	-	-	-	34 (0- 63)	17 (0- 49)	48 (37- 63)
	AE	-1431.86	443	0.90	1	0.34	-1.10	54 (40- 64)	-	46 (36- 60)
PC2 age-group 3	ACE	-1352.70	408	-	-	-	-	58 (23- 69)	0 (0- 29)	42 (31- 55)
	AE	-1352.70	409	0.00	1	1.00	-2.00	58 (45- 69)	-	42 (31- 55)

In the structural equation modelling, an ACE model (model) was fitted to the observed phenotypic variance in unadjusted PC1 and PC2 values at different age groups based on maximum likelihood estimation. For each trait, three nested models were compared to the full ACE model, taking into account different causal factors: AE (additive genetics and unshared environmental factors), CE (shared and unshared environmental factors) and E (unshared environmental factors). Model fit for the full and respective reduced model were compared in a likelihood ratio test. Significance of the likelihood ratio test (χ^2 p-value) is determined in a chi-square statistic based on the difference in twice the negative logarithm of the likelihood (Δ-2LogL) and difference in degrees of freedom (Δdf) between the compared models. In addition, the Akaike's information criterion (AIC) gives a measure of parsimony of a reduced model in comparison to the full model. For each phenotype the ACE model fit and nested models with a better model fit (highlighted in grey) are shown. Model comparison is only given for nested models as they are compared to the full (ACE) model. Estimated variances explained by the specific causal factors (A= additive genetics, C= shared environment and E= unshared environment) are given with 95 % confidence intervals (95% CI) for each model.

Discussion

In this chapter, the heritability of hearing ability with age was estimated in female twin volunteers of the TwinsUK register. Correlation in hearing ability within MZ twin pairs exceeded DZ twin pair correlations, indicating a genetic component influencing the variance in ARHI.

Two methods were applied to estimate the heritability of hearing ability, comparison of intraclass correlations between MZ and DZ twin pairs based on Falconer's formula and SEM based on the classical twin model. SEM, in comparison to Falconer's formula, gives a measure of how likely the observed data is under a specific model and calculates confidence intervals for each parameter based on the model fit. In addition, covariates like age or gender could be incorporated in the model; however, it was decided to adjust for age previous to analysis. Although SEM based on the classical twin model appears more advanced than heritability estimates based on Falconer's formula, both methods were applied to test for consistency in the results. In general, heritability estimates obtained by SEM and Falconer's formula were very consistent for all phenotypes but age-adjusted PC2 (Table 14). In case of age-adjusted PC2, heritability based on Falconer's formula exceeded the confidence intervals for the SEM estimate for A (Table 14). Furthermore, the comparison of intraclass correlation coefficients between MZ and DZ twin pairs, as applied in Falconer's formula, helped to decide between an ACE and ACD model [4].

When heritability estimates were compared for unadjusted and age-adjusted PC1 and PC2, a general trend towards higher heritability (A) and decreased effect of common environmental factors (C) on variance in ARHI could be recognized for age-adjusted values (Table 15). This trend has been described previously for twin modelling [14] and reasons can be proposed for it. The twin model aims to decompose trait variance into variance components based on within twin pair correlation. Age is a common factor shared within both MZ and DZ twin pairs and will therefore be interpreted as a common environmental factor in twin modelling if age has an effect on the trait. Furthermore, variance component estimates (A, C, E) and ICCs are expressed in proportion to the phenotypic variance in the study sample. An increased variance due to lack of age-adjustment could therefore lead to decreased variance component estimates. Table 13 shows an increased variance for unadjusted PC1 values compared to age-adjusted values, however, this does not account for variance in PC2. Accordingly, in the SEM heritability was increased for age-adjusted PC1 values compared to unadjusted PC1, but not for age-adjusted PC2 values (Table 15). The heritability for age-adjusted PC1

values (A=61% (95% CI: 54- 67)) were comparable to the estimates determined for a mixed gender sample by *Huyghe et al* [10](h^2= 66.3%) (Table 15). However, heritability estimates for PC2 (A=56% (95% CI: 49- 63) were considerably higher in TwinsUK compared to *Huyghe et al* [10] (h^2=27.2%)(Table 15).

When comparing heritability estimates for different age-groups, estimates for A increased from 57% to 77% (Table 18) with increasing age in SEM. The same trend could be detected in the heritability estimates based on Falconer's formula. Here, H^2 increased from 28% to 74% (Table 17) in correlation with age. The same increase in heritability was determined for PC2: A increased from 49% to 58% (Table 18), while H^2 estimates first increased from 44% to 76% with higher age (Table 17). The change in variance components on ARHI with increasing age has previously been studied by *Karlsson et al* [7]. In this male twin study, the effect of unique environmental factors (E) showed an increasing effect on ARHI while, broad sense heritability decreased from 100% to 47.4% [7]. This difference in change in heritability with age might be a gender specific effect, as suggested by Gates [9], who justified the higher aggregation of hearing in female relatives by more limited exposure of women to occupational noise compared to men. This hypothesis is further supported by the low exposure to environmental risk factors for TwinsUK as reported in chapter 2. However, it should also be considered that ARHI might be influenced by different genetic factors in men and women, which show different impacts on hearing at different stages in development. In comparison to a female twin sample of similar age range (age range: 63-76 years)[8], heritability estimates for PC1 at the highest age group (A=77% (95% CI: 69- 83**,** age range: 66-86) were highly comparable to the ones for the BEHL (A=75% (95% CI: 67-81)) (Table 18). In conclusion, decreased risk factor exposure (i.e. to occupational noise) in women might reduce the effect of unique environmental factors on ARHI and therefore increase heritability estimates compared to male samples.

The increase in heritability with increasing age might be interpreted in different ways. First, the impact of genetic factors on ARHI could rise due to changes in gene expression or accumulation of genetic mutations with higher age. Secondly, the reduced impact of environmental factors on ARHI with increasing age could indicate that vulnerability to environmental risk factors is higher at a younger age. Similar has been reported for mouse models, where noise exposure had a higher effect on hearing ability with age if the exposure occurred during early rather than late development [15]. In general, recruiting study samples at an age range at which heritability of hearing

ability is maximised might give a potential power advantage for future genetic association studies.

The classical twin model is based on assumptions that were not addressed in the analysis of this chapter. The twin sample studied here has been shown to be representative of the UK singleton population [16]. Furthermore, similar hearing ability could be determined for comparable singleton cohorts (chapter 2). The high age of onset for ARHI should prevent assertive mating due to hearing loss. Twin studies are often criticised in respect to the assumption of equally shared environments within MZ and DZ twin pairs with MZ twin pairs being assumed to be treated more similarly than DZ twin pairs. This would imply that MZ twins are exposed to a higher extent of shared environment. Studying DZ twins, who have falsely been labelled as MZ twins, could disprove this hypothesis [4]. If these mislabelled pairs shared a higher amount of shared environment than their true zygosity suggests, the assumption of equal environments would be violated. However, testing for equally shared environments was beyond the scope of this study. It is tempting to compare the findings of this heritability analysis to previous heritability analysis on ARHI, however, heritability is a population specific estimate [6], dependent on the population specific trait prevalence, environment and genetic variation. The comparison of heritability estimates for different population samples is therefore limited and should be interpreted with caution. Furthermore, this study was focused on a purely female sample, and can thus not make predictions on heritability in a mixed gender or male population. In addition, power calculation for heritability studies based on twin models suggests sample sizes of at least 200 twin pairs [17] to reach significant statistical power. In consideration of this, the analysis by age groups used therefore relatively limited sample sizes ($n \geq 415$ subjects).

In this chapter, the proportion of variance in ARHI explained by additive genetic, shared and unshared environmental factors, was estimated based on twin modelling. The presented results show that hearing ability with age is determined by up to 61% by genetic variants, while the remaining 39% of variance in ARHI are determined by unique environmental exposures. This suggests that shared environmental factors play only a minor to no role in hearing ability with age. Furthermore, a rising heritability of ARHI with increasing age is reported. In conclusion, the moderate heritability determined for age-adjusted PC1 and PC2 in TwinsUK females supports the use of this sample for further analysis on a genome-wide level to identify the genetic variants

involved in ARHI. The increase in heritability with age for PC1 and PC2 suggests recruitment of older (>65 years) subjects for future genetic association studies of ARHI.

References

1. Falconer DS (1981) Introduction to quantitative genetics: Longman.
2. Galton F (1876) The History of Twins, as a Criterion of the Relative Powers of Nature and Nurture. The Journal of the Anthropological Institute of Great Britain and Ireland 5: 391-406.
3. Siemens HW (1924) Die Zwillingspathologie. Zeitschrift für Induktive Abstammungs- und Vererbungslehre 35: 311-312.
4. Rijsdijk FV, Sham PC (2002) Analytic approaches to twin data using structural equation models. Briefings in Bioinformatics 3: 119-133.
5. Neale MC, Boker SM, Xie G, Maes HH (2006) Mx: Statistical Modeling.7th Edition.
6. Visscher PM, Hill WG, Wray NR (2008) Heritability in the genomics era [mdash] concepts and misconceptions. Nat Rev Genet 9: 255-266.
7. Karlsson KK, Harris JR, Svartengren M (1997) Description and primary results from an audiometric study of male twins. Ear Hear 18: 114-120.
8. Viljanen A, Era P, Kaprio J, Pyykkö I, Koskenvuo M, et al. (2007) Genetic and Environmental Influences on Hearing in Older Women. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences 62: 447-452.
9. Gates GA, Couropmitree NN, Myers RH (1999) Genetic associations in age-related hearing thresholds. Arch Otolaryngol Head Neck Surg 125: 654-659.
10. Huyghe JR, Van Laer L, Hendrickx JJ, Fransen E, Demeester K, et al. (2008) Genome-wide SNP-based linkage scan identifies a locus on 8q24 for an age-related hearing impairment trait. Am J Hum Genet 83: 401-407.
11. Christensen K, Frederiksen H, Hoffman HJ (2001) Genetic and environmental influences on self□reported reduced hearing in the old and oldest old. Journal of the American Geriatrics Society 49: 1512-1517.
12. Neale M, Cardon LR (1992) Methodology for genetic studies of twins and families: Kluwer Academic Pub.
13. Akaike H (1974) A new look at the statistical model identification. Automatic Control, IEEE Transactions on 19: 716-723.

14. Snieder H (2000) Path analysis of age-related disease traits. In: Spector TD, Snieder H, MacGregor AJ, editors. Advances in twin and sib-pair analysis. London

Oxford ; New York: Greenwich Medical Media ;

Distributed worldwide by Oxford University Press. pp. xv, 266 p.

15. Kujawa SG, Liberman MC (2006) Acceleration of age-related hearing loss by early noise exposure: evidence of a misspent youth. The Journal of Neuroscience 26: 2115-2123.

16. Andrew T, Hart DJ, Snieder H, de Lange M, Spector TD, et al. (2001) Are twins and singletons comparable? A study of disease-related and lifestyle characteristics in adult women. Twin Res 4: 464-477.

17. Martin NG, Eaves LJ, Kearsey MJ, Davies P (1978) The power of the classical twin study. Heredity 40: 97-116.

Chapter 4: Validation and heritability of the speech in noise ratio test

Abstract

Pure-tone audiometry is accepted as the gold standard hearing tests by most audiologists. Still, pitch perception at different frequencies in a quiet environment can only make limited predictions about speech perception in a noisy environment. Communication difficulty in a noisy environment is a major symptom of ARHI. Various speech intelligibility tests have been developed to measure speech perception in real life situations.

Speech intelligibility in noise was assessed using a web-based speech-in-noise test kindly provided by Action on Hearing Loss. Heritability of speech reception in noise was estimated in a heritability analysis based on the classical twin model. The speech-in-noise test was validated against pure-tone audiometry in 448 subjects having completed both hearing tests. Shared heritability for hearing ability as measured by both tests was determined in a bivariate correlated factors model.

Speech reception thresholds were collected for 1909 subjects (1684 females and 225 males) from the TwinsUK register. Additive genetic factors accounted for 34% (95% CI: 26-42) of the variance in speech reception thresholds, while environmental factors unshared within twin pairs explained the remaining variance (E=66% (95% CI: 58- 75). At a speech reception threshold of -9.25 dB, the speech-in-noise test showed a sensitivity and specificity of 88.24% and 80.05% in comparison to moderate hearing loss defined as a pure-tone average ≥40dB HL. Shared additive genetic factors explained up to 68.8% of phenotypic correlation between speech reception thresholds and pure-tone averages.

The sensitivity and specificity of the speech-in-noise hearing test in comparison to pure-tone audiometry supports the use of this web-based test as a predictor for moderate hearing loss. The proportion of shared heritability between both tests suggests the use of both hearing phenotypes (speech reception in noise and pitch perception at different frequencies) as validation datasets for each other in future genetic studies.

Introduction

Advanced ARHI can cause severe communication difficulties resulting in social isolation [1] and incapability to work. Despite the high impact of ARHI on daily life activities, many affected individuals do not recognize and/ or acknowledge their hearing loss until it is well advanced. As shown in chapter 2, self reported hearing loss showed a sensitivity of 86.6% and a specificity of 75.5% to correctly identify individuals with moderate hearing loss (pure-tone average ≥ 40 dB HL). To better understand the factors leading to the hearing loss with advanced age, large samples need to be collected. Previous genome-wide association studies of complex traits like ARHI suggests that ~5000 subjects (2000 cases and 3000 controls) might be required to reliably identify genetic variants associated with disease [2]. Reaching sample sizes of such magnitude for studies of ARHI seem illusionary when measuring hearing ability using standard pure-tone audiometry. The collection of large samples for studies of ARHI has been impaired by various factors: decreased mobility of elderly subjects to attend test centres; length of test procedure (air conduction pure-tone audiometry without masking takes ~15 minutes per tested individual) and availability of trained testers and equipment (i.e. audiometer, headphones, sound insulated environment). The optimal screening hearing test would therefore allow fast and efficient medical testing from at home requiring limited assistance by medically trained personal. With increased access of elderly users to the Internet, the worldwide web has often been suggested as a future platform for health care information and possibly medical tests [3,4,5,6].

Difficulty understanding other individuals in a noisy environment is one of the major symptoms reported by individuals with ARHI. The standard hearing test, air-conduction pure-tone audiometry, tests pitch perception at different frequencies (0.125-8.0 kHz) in a quiet environment. Although the pure-tone audiogram is the gold standard test to assess hearing ability, it can only make predictions concerning hearing performance in real life situations. Subjects with word recognition difficulties due to neural hearing loss might go unnoticed according to their pure-tone audiogram. Hearing tests which access word recognition rather than pitch perception have therefore long been used in audiology as an addition to the pure-tone audiogram [7].

Most speech perception hearing tests are based on phonemic word groups or whole sentences, which are presented to the listener at various sound intensities and asked to be repeated by the listener [8,9]. Using complete sentences as speech material allows the tested individual to guess single words to form a meaningful sentence. In

addition, many speech perception tests are limited to a defined selection of speech material [10,11]. An increased learning effect due to repetition of speech material becomes thus more likely. Numbers or digits provide a unique opportunity as test material as they can be combined independently of each other. Furthermore, numbers are more easily entered at a keyboard as part of an automated test procedure in contrast to words or sentences as a response.

The majority of hearing tests are designed for conduction in a quiet environment or ideally in a sound isolated room [12]. However, this does not reflect the challenges encountered by hearing impaired subjects on a daily basis. To mirror normal life conditions, background noise would be required during the test. Noise at frequency ranges similar to the range of the speech signal would give the highest masking effect and thereby allow the test to be more challenging and authentic for the listener.

The considerations presented here were incorporated in the design of a digits-in-noise screening test for telephone usage in the Netherlands [13,14]. This test has since been used and described for the Dutch national screening program [15], was adapted for internet use [16] and different languages [17,18]. Speech reception thresholds (SRTs) as measured by the digits-in-noise test [19] were highly correlated (r=0.82-0.90) with the standard speech-in-noise test SRTs obtained using sentences as speech material [8] in 23 subjects. Although this test has previously been compared to other speech-in-noise tests [13,19] it has not been published showing validation against standard pure-tone audiometry.

Materials and Methods

Speech in noise test study samples

Recruitment for the speech-in-noise online hearing test was limited to volunteers from the TwinsUK cohort with registered email addresses (n=4811), including adult (≥18 years) subjects of both genders. Subjects with valid email addresses were assumed to have regular access to the Internet and therefore deemed suitable for this web-based test. Recruitment was conducted between March and September 2011. All eligible volunteers were invited to take part in the test via a recruitment email including instructions concerning the test procedure and how to access the test. Test results were saved using a unique identification number per individual.

Speech reception threshold data collection

The speech-in-noise test measures speech intelligibility against increasing background noise. The test used in this study was originally developed for telephone use [13] and converted for web based use via loud speakers by Action on Hearing loss [20]. During the test, combinations of digit-triplets (i.e. 3-7-9 spoken as “three seven nine”) were presented at a constant sound intensity against a variable intensity of noise spectrum based on the long-term speech spectrum [13]. At the beginning of each test procedure, volunteers were asked to set a comfortable speech intensity according to their own hearing ability. This speech volume was kept constant throughout the test while the noise intensity was allowed to vary. Triple digits were uttered by a female voice. An adaptive up-down procedure was applied according to which noise intensity was increased or decreased in steps of 2 dB depending upon correct or incorrect identification of all three digits, respectively. Per test, 26 digit triplets were presented and the SRT, defined as the threshold at which 50% of presentations were identified correctly, was determined as the average SNR over all 26 presentations. The SRT was measured for both ears together (binaural) via computer speakers and subjects were asked to perform the test on their own in a quiet environment. Test results were saved under the unique identification number and date of birth provided for each subject as well as time and date of test conduction. Volunteers were allowed to repeat the test whereas each test result was saved with its respective time and date of conduction. In the Dutch screening test only a slight learning effect could be registered between the first and following 24 tests in naïve listeners, with the mean SRT being 1.3 dB worse for the first test compared to the 4 following measurements [19]. Therefore, if a subject completed the speech-in-noise test several times, the most recent test result was used.

The sound intensity for both speech and noise signals was measured in decibel and thus on a logarithmic scale (see chapter 2). According to calculus rules, the ratio between two logarithms is expressed as their difference. The SNR is therefore calculated as the difference in speech and noise sound intensity and expressed in decibels (Equation 14).

$$SNR[dB] = 20 \times \log_{10}\left(\frac{S}{n}\right) = \mathrm{s}[dB] - n[dB]$$

[Equation 14 Speech-to-noise ratio]

With SNR= speech-to-noise ratio, s=speech sound intensity and n= noise sound intensity, [dB]= expressed in decibels.

The SRT ranged between +8 dB and -14 dB, where a high value indicated hearing difficulties (speech could only be heard at a low background noise) and a low value

represented good speech reception even at high masking noise. To enable simpler interpretation of test results, the SRT was converted to a score from 0 to 11, which was inversely correlated with the SRT [21]. A low score indicated hearing loss while a high score indicated good speech reception in noise (Table 19). The relationship between SRTs and scores can be expressed as SRT= 8+ (-2x score).

Table 19 Conversion chart for score and SRT

Hearing ability in noise	score	SRT [dB]
low (score<7.5)	0	8 dB
	1	6 dB
	2	4 dB
	3	2 dB
	4	0 dB
	5	- 2 dB
	6	- 4 dB
moderate (score >7.5 &< 8.5)	7	- 6 dB
	8	-8 dB
good (score ≥ 8.5)	9	-10 dB
	10	-12 dB
	11	-14 dB

The web-based speech-to-noise ratio test measured hearing ability in speech reception thresholds (SRTs) and scores. Poor hearing ability was indicated by a low score and high SRT, whereas good hearing ability was reflected by a high score and low SRT.

Original SRTs followed a skewed distribution. To ensure overall positive SRT values and thereby facilitate transformation a constant of 20 was added to all SRTs. The resulting positive SRT values were transformed to normality by dividing 1 by the square of the positive SRTs (Equation 15).

$$transformed\ SRT = \frac{1}{(SRT+20)^2}$$

[Equation 15, transformation of SRT values to normality]

with SRT= speech reception threshold

Linear regression analysis adjusted for twin relatedness was used to test whether age and gender had a significant effect on transformed SRT values.

Univariate heritability estimates for SRTs

Univariate heritability estimates of the SRT were based on the classical twin model as described in detail in chapter 3. Heritability estimates were determined using maximum likelihood estimation based on a full ACE model (ACE). Model fit for reduced models (CE, AE, E) were compared to the model fit for the full model in a likelihood ratio test

[22]. In addition to structural equation modelling using maximum likelihood estimation, broad sense heritability estimates were derived using Falconer's formula [23] (chapter 3).

Univariate heritability analysis was performed for transformed SRTs and age-adjusted residuals of the latter. Analysis was performed for the complete sample with speech-in-noise test data (males and females) and for female subjects only.

Study sample with pure-tone audiogram and speech-in-noise test data

To compare and validate the SRT against standard pure-tone audiometry, all subjects with pure-tone audiogram and speech-in-noise test data were selected. Individuals with pure-tone audiograms were chosen from the original dataset remaining after quality control as described in chapter 2 (n=1303). The pure-tone audiometry (n=1303) and SRT (n=1909) datasets were merged by the unique identification number for each subject.

Validation of speech-in-noise test against pure-tone audiometry

For descriptive comparison of SNR and pure-tone audiogram measures, association was tested with PC1, PC2, PTA and age-adjusted PTA. To validate the SRT against pure-tone audiometry, the sensitivity and specificity for different SRTs against moderate hearing loss (HL) defined as a pure-tone average (calculated over frequencies 0.125-8 kHz) ≥ 40 dB HL were obtained. The sensitivity of the SRT represents the proportion of subjects correctly diagnosed with moderate HL (according to the PTA≥40 dB HL) at the respective SRT, whereas specificity corresponds to the proportion of volunteers without HL that were correctly identified as healthy hearing at the respective SRT [24]. The result of this analysis for various SRTs is summarised in a receiver operating curve (ROC), which plots (1-specificity) against the sensitivity calculated for different SRT.

Bivariate heritability of SRT and pure-tone audiogram measures

To determine shared causal factors between both hearing tests, bivariate structural equation modelling based on a correlated factors model was performed for PTAs and SRTs. The correlated factors model assumes no known direction of causality between both variables [25]**.** This model determines the correlation (r_a, r_C, r_e) between variance components (A, C, E) estimated for both measures of hearing ability and can therefore

give an estimate of shared variance components (i.e. shared heritability) between both measures (Figure 17).

The bivariate correlated factors model (Figure 17) was fitted to transformed SRTs and PTA values as well as age-adjusted transformed SRT residuals and age-adjusted PTA residuals. For both bivariate models, the full ACE model was compared to a reduced AE model based on a likelihood ratio test [26] (chapter 3).

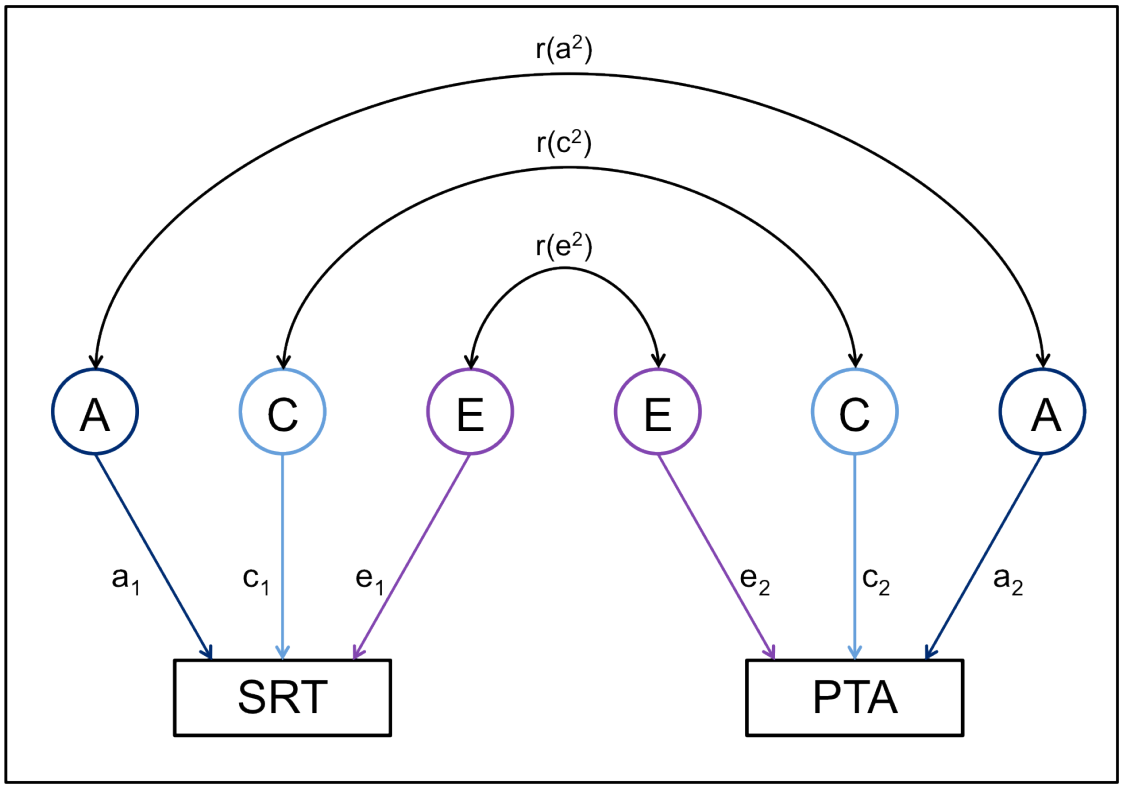


Figure 17 Bivariate correlated factors model pathway

The bivariate correlated factors model describes the univariate heritability estimates for two observed phenotypes (i.e. SRT and PTA results) and determines the correlation ($r(a^2)$, $r(c^2)$ and $r(e^2)$) between the corresponding estimates (A, C, E). The two observed phenotypes are indicated by black boxes. Univariate variance component analysis is based on the classical twin model. The single headed arrows represent univariate path coefficients, measuring the effect of the latent variables on the phenotype. The phenotypic variance attributed to the specific variance components (A, C, E) can be calculated as the square of the respective path coefficients (i.e. $A=a_1^2$). Double-headed arrows indicate correlations between the estimated variance components (A, C and E) between both phenotypes.

The results of the bivariate heritability analyses were further used to calculate the percentage of phenotypic correlation due to shared additive genetic effects for the corresponding phenotype pairs. This calculation can be completed by tracing the path of the additive genetic effects from one phenotype to the next (path: $a_1 \times r(a^2) \times a_2$)

according to the bivariate correlated factors model pathway (Figure 17) and dividing the total by the phenotypic correlation coefficient (Equation 16).

$$r(P_A) = \frac{\sqrt{A_{SRT}} \times r(a^2) \times \sqrt{A_{PTA}}}{r(SRT,PTA)} = \frac{a_1 \times a_2 \times r(a^2)}{r(SRT,PTA)}$$

[Equation 16 Percentage of phenotypic correlation due to shared additive genetics]

with A_{SRT}=heritability of hearing ability as measured by SRT, A_{PTA}=hearing ability as measured by pure-tone average, $r(a^2)$= correlation of SRT and PTA heritability estimates and r(SRT, PTA)= phenotypic correlation coefficient for correlation between SRT and PTA measures of hearing ability.

Results

SRT study sample

Between March and September 2011, 1909 individuals from TwinsUK were recruited to test their hearing online using the web-based speech-in-noise test. Considering that 4811 individuals were invited via email, this corresponds to a response rate of 39.68%. The SRT sample included 349 MZ twin pairs, 179 DZ twin pairs and 853 unpaired twins. For this test, both female and male twins were recruited, with 1684 female and 225 male volunteers having completed the speech-in-noise test. Age of the participants ranged from 19 to 85 years, giving a mean age of 54.63 years ±12.79 standard deviation. Original SRTs followed a skewed distribution (appendix chapter 4, Figure 48) that could not be transformed to normality using a log-transformation. A histogram of the transformed SRT values can be seen in appendix chapter 4, Figure 49.

Although males showed slightly lower SRTs (n=225; coef=-0.0003±0.0002 se) this effect was not significant (p=0.171). However, chronological age at the test date explained 16.75% of the variance in transformed SRTs and was significantly associated with transformed SRT values (p=0.000). Increasing age had a negative effect on transformed SRTs (coef=-0.000011 ± 6.34×10^{-6} se) (appendix chapter 4, Figure 47). To adjust for this effect of age, age-adjusted SRT residuals were taken. A histogram of the transformed age-adjusted SRT residuals can be seen in appendix chapter 4, Figure 50. There was no significant difference between speech-in-noise test scores and original, transformed or age-adjusted SRTs between MZs, DZs and unpaired twins (ANOVA, p>0.05). Speech-in-noise ratio test results were listed by zygosity (Table 20) and gender (Table 21).

Table 20 Speech-in-noise test samples by zygosity

zygosity	n	gender F/M	age		score mean ±SD	SRT mean ±SD	transformed SRT mean ±SD	age-adjusted SRT mean ±SD
			range	mean ±SD				
MZ	698	628/ 70	20-85	53.28 ±14.15	9.1301 ±1.0379	-10.2602 ±2.0759	0.0116 ±0.0036	-3.419 E-05 ±0.0033
DZ	358	330 / 28	28-78	57.24 ±10.20	9.0939 ±1.0844	-10.1879 ±2.1689	0.0115 ±0.0036	0.0003 ±0.0033
unpaired twins	853	726/ 127	19-84	54.63 ±12.44	9.0757 ±1.1045	-10.1514 ±2.2089	0.0114 ±0.0035	-0.0001 ±0.0032
total	1909	1684/ 225	19-85	54.63 ±12.79	9.0990 ±1.0765	-10.1980 ±2.1530	0.0115 ±0.0035	-3.044 E-12 ±0.0032

This table summarizes the characteristics of the all subjects with speech-in-noise test data by zygosity. The sample was divided into three subsamples by zygosity of the participants. Each subsample was characterized by the number of subjects (n), gender, mean chronological age and age range at speech-in-noise test. Furthermore, speech-in-noise test scores, SRTs, transformed as well as age-adjusted SRT values are summarised by zygosity as mean ± standard deviation (SD) from the mean.

Table 21 Speech-in-noise test samples by gender

gender	n	zygosity: DZ MZ unpaired twins	age		score mean ±SD	SRT mean ±SD	transformed SRT mean ±SD	age-adjusted SRT mean ±SD
			range	mean ±SD				
female	1684	330 628 726	19-82	54.52 ±12.69	9.1149 ±1.0638	-10.2297 ±2.1277	0.0115 ±0.0035	3.902 E-05 ±0.0032
male	225	28 70 127	21-85	55.44 ±13.51	8.9803 ±1.1626	-9.9606 ±2.3253	0.01101 ±0.0036	-3.000 E-04 ±0.0033
total	1909	358 698 853	19-85	54.63 ±12.79	9.0990 ±1.0765	-10.1980 ±2.1530	0.0115 ±0.0035	-3.044 E-12 ±0.0032

This table summarizes the characteristics of all subjects with speech-in-noise test data by gender. The sample was divided into female and male participants. Both female and male subjects were characterized by the number of subjects (n), zygosity, age range and mean chronological age at speech-in-noise test. Speech-in-noise test scores, SRTs, transformed as well as age-adjusted SRT values were summarised as mean ± standard deviation (SD) from the mean.

Univariate heritability of speech reception in noise

Univariate heritability estimates were calculated for transformed and age-adjusted SRT residuals using two methods, Falconer's formula and structural equation modelling based on the classical twin model.

Broad sense heritability estimates according to Falconer's formula ranged between H^2=32% for transformed SRTs and H^2=36% for age-adjusted SRT residuals (Table 22). Results of the structural equation modelling suggested that speech intelligibility against background noise was primarily influenced by exposure to environmental factors not shared within twin pairs (E: 66-80%) (Table 23). The AE model gave a more parsimonious model fit in comparison to the full ACE model for all SRT phenotypes. While broad sense heritability estimates (H^2) increased after age-adjustment of SRT values using Falconer's formula, the reverse effect could be seen in structural equation modelling, where heritability decreased from A=34% (95% CI: 26- 42**)** to A=20%(95% CI: 10- 29). As previously reported, age-adjustment reduced the estimated effect of common environmental factors on phenotypic variance [27] from C=6% (95% CI: 0- 32) to C=0% (95% CI: 0-19).

The heritability of SRT values in a purely female sample was investigated. In the structural equation modelling with female subjects only, the AE model fitted the observed variance in age-adjusted SRT residuals best. Variance component estimates ranged from 19% (95% CI: 8- 28) for additive genetic factors (A) to 81% (95% CI: 72- 92) for unshared environmental factors within twin pairs (E) (Table 23).

Table 22 Intraclass correlation coefficients and heritability estimates for transformed and age-adjusted SRT residuals

phenotype	ICC_{MZ}	ICC_{DZ}	H^2	A% (95% CI)
transformed SRT	0.34	0.18	32%	34 (26- 42)
age-adjusted SRT	0.20	0.02	36%	20 (10- 29)

The intraclass correlation coefficients for MZ (ICC_{MZ}) and DZ (ICC_{DZ}) twin pairs were determined for each phenotype and broad sense heritability (H^2) calculated as twice the difference in ICCs for MZs and DZs ($H^2=2x(ICC_{MZ} - ICC_{DZ})$). For comparison, the percentage of phenotypic variance explained by additive genetics (A%) determined in univariate structural equation modelling (Table 23) for the respective phenotype was added with 95% confidence intervals.

Table 23 Results of the structural equation modelling used to estimate the influence of A, C and E on speech-in-noise ratio

phenotype	model fit			model comparison				univariate estimates % (95% CI)		
	model	-2LogL	df	$\Delta \chi^2$	Δ df	p-value	AIC	A	C	E
transformed SRT	ACE	-16186.77	1902	-	-	-	-	28 (0- 42)	6 (0- 32)	66 (58- 76)
	AE	-16186.61	1903	0.16	1	0.69	-1.84	34 (26- 42)	-	66 (58- 75)
age-adjusted SRT residuals	ACE	-16505.17	1902	-	-	-	-	20 (0- 29)	0 (0-19)	80 (71- 90)
	AE	-16505.17	1903	0.00	1	1.00	-2.00	20 (10- 29)	-	80 (71- 90)
age-adjusted SRT residuals (females only)	ACE	-14565.20	1677	-	-	-	-	19 (0- 28)	0 (0- 22)	81 (72- 92)
	AE	-14565.20	1678	0.00	1	1.00	-2.00	19 (8- 28)	-	81 (72- 92)

In the structural equation modelling an ACE model was fitted to the observed phenotypic variance in transformed and age-adjusted SRT residuals based on maximum likelihood estimation. For each trait, three nested models were compared to the full ACE model, taking into account different causal factors: AE (additive genetics and unshared environmental factors), CE (shared and unshared environmental factors) and E (unshared environmental factors). Model fit for the full and respective reduced model were compared in a likelihood ratio test. Significance of the likelihood ratio test (p-value) is based on a chi-square statistic ($\Delta\chi^2$) with 1 or 2 degrees of freedom (Δdf). In addition, the Akaike's information criterion (AIC) gives a measure of parsimony of a reduced model in comparison to the full model. For each phenotype the ACE model fit and nested models with a better model fit (highlighted in grey) are shown. Model comparison is only given for nested models as they are compared to the full (ACE) model. Estimated variances explained by the specific causal factors (A= additive genetics, C= shared environment and E= unshared environment) are given with 95 % confidence intervals (95% CI) for each model.

Table 24 Description of samples with speech-in-noise and pure-tone audiogram test data

demographics				pure-tone audiogram phenotypes				speech reception phenotypes		
zygosity	n	age at audiogram		PC1	PC2	PTA	age-adjusted PTA	SRT	trans-formed SRT	age-adjusted SRT residuals
		range	mean ±SD							
MZ	142	41- 80	58.92 ±8.65	-0.213 ±1.735	0.238 ±1.345	20.337 ±8.921	0.460 ±7.322	-10.395 ±1.612	0.012 ±0.003	0.001 ±0.003
DZ	116	47- 79	61.07 ±6.35	-0.309 ±1.737	-0.022 ±1.246	20.598 ±9.030	-0.573 ±7.474	-10.160 ±2.021	0.011 ±0.004	0.001 ±0.004
unpaired twins	190	41- 80	60.35 ±8.16	-0.251 ±2.012	0.008 ±1.381	20.745 ±9.644	0.006 ±8.587	-10.061 ±1.903	0.011 ±0.003	0.000 ±0.003
Total	448	41- 80	60.09 ±7.93	-0.254 ±1.854	0.073 ±1.338	20.578 ±9.243	4.7E-10 ±7.913	-10.193 ±1.850	0.011 ±0.003	0.000 ±0.003

Subjects with speech-in-noise test data and pure-tone audiometry were described by demographic measures, pure-tone audiogram phenotypes and speech-in-noise test phenotypes. The sample was divided into three subsamples by zygosity of the participants (monozygotic twins (MZ), dizygotic twins (DZ) and unpaired twins. Each subsample was characterized by the number of subjects (n), mean chronological age and age range at pure-tone audiogram. Pure-tone audiogram and speech-in-noise test phenotypes are summarised as mean and standard deviation from the mean (SD) (PC1=principal component 1, PC2=principal component 2, PTA=pure-tone average, SRT=speech reception threshold). All subjects presented in this table were female.

Study sample with SRT and pure-tone audiogram

For the speech-in-noise test validation and bivariate heritability analysis between SRT and pure-tone audiogram measures, all individuals having completed both speech-in-noise test and the pure-tone audiogram were selected. In total, 448 subjects completed both tests, comprising 142 MZs, 116 DZs and 190 unpaired twins. This study sample included only female subjects, as recruitment for the pure-tone audiogram had been limited to female volunteers only. Age at audiogram ranged from 41-80 years (mean age: 60.09± 7.93 SD). Both hearing tests had been performed within ≤2 years of each other. The mean difference in age at both tests amounted to 0.58 years± 0.59 SD. The results for different pure-tone audiogram measures (PC1, PC2, PTA and age-adjusted PTA residuals) and speech-in-noise measures (SRT, transformed SRT and age-adjusted SRT residuals) are summarised by zygosity in Table 24. As shown above for the speech-in-noise test and previously for the audiogram dataset (chapter 2), there was no significant difference in results between MZs and DZs.

Correlation between speech-in-noise test and pure-tone audiogram measures

To determine the relationship between pure-tone audiogram and SRT measures, the correlation between test results was measured. The resulting correlation coefficients

are shown in Table 25. The highest correlation could be observed between SRTs and PTA results (unadjusted and age-adjusted residuals). Transformed SRTs (transformed SRTs and age-adjusted SRT residuals) were negatively correlated with audiogram phenotypes, as a result of the transformation of SRTs to normality. The strongest absolute correlation for transformed SRT values was seen with PTAs. To adjust for the effect of age, the association between age-adjusted SRT residuals and age-adjusted PTA residuals was determined by correlation analysis. Age-adjustment reduced the correlation coefficient from r=-0.4519 to r=-0.3280 (Table 25).

Table 25 Correlation matrix for different pure-tone audiogram and speech-in-noise phenotypes

Correlation coefficients between speech in noise and pure-tone audiogram phenotypes			
pure-tone audiogram phenotypes	speech-in-noise phenotypes		
	SRT	transformed SRT	age-adjusted SRT residuals
PC1	0.2995	-0.2314	-0.2568
PC2	0.1795	-0.1628	-0.1779
PTA	0.5249	-0.4519	-0.3388
age-adjusted PTA residuals	0.3942	-0.3048	-0.3280

The above table lists correlation coefficients between different pure-tone audiogram and speech reception thresholds (SRTs). Pure-tone audiogram phenotypes include: Principal component 1 (PC1) and 2 (PC2) as well as a pure-tone average (PTA) calculated as the mean pure-tone threshold measured over 0.125-8.0 kHz.

Validation of SRT against pure-tone audiometry

To validate the speech-in-noise test, test results were compared to hearing impairment as measured by the standard hearing test, pure-tone audiometry. Moderate hearing impairment was defined as a PTA≥ 40 dB HL based on thresholds adapted from the WHO [28]. This standard criterion was used as the reference measurement to determine the sensitivity and specificity of the SRT to correctly identify or reject subjects for moderate hearing loss. The analysis was performed for both SRT and transformed SRT values and depicted in receiver operating curves (Figure 18 and Figure 19). At an SRT of -9.25 dB, the speech in noise test showed 88.24% sensitivity and 80.05% specificity to correctly identify moderate hearing loss. The same sensitivity and specificity was determined for a transformed SRT value of 0.0087. The area under the curve measured 0.9238 with a standard error of 0.0245 (95% CI: 0.8758-0.9719) for both SRT and transformed SRT values (Figure 18 and Figure 19).

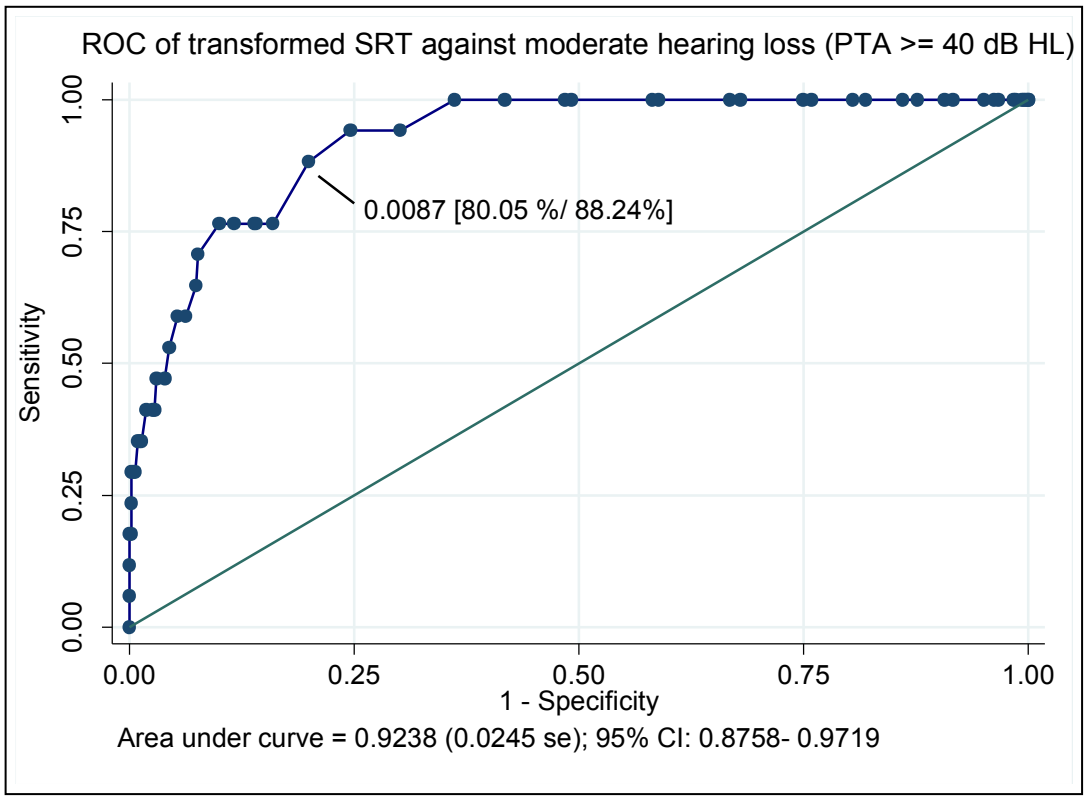


Figure 18 Receiver operating curve of transformed SRTs against moderate hearing loss

The receiver operating curve (ROC) plots the specificity versus the sensitivity of the transformed speech reception threshold (SRT) in comparison to hearing loss as measured by the pure-tone average ≥ 40 dB HL. At a transformed SRT value of 0.0087, a sensitivity and specificity of 88.24% and 80.05% was observed, respectively. The area under the curve is presented with standard error (se) and 95% confidence intervals (95% CI).

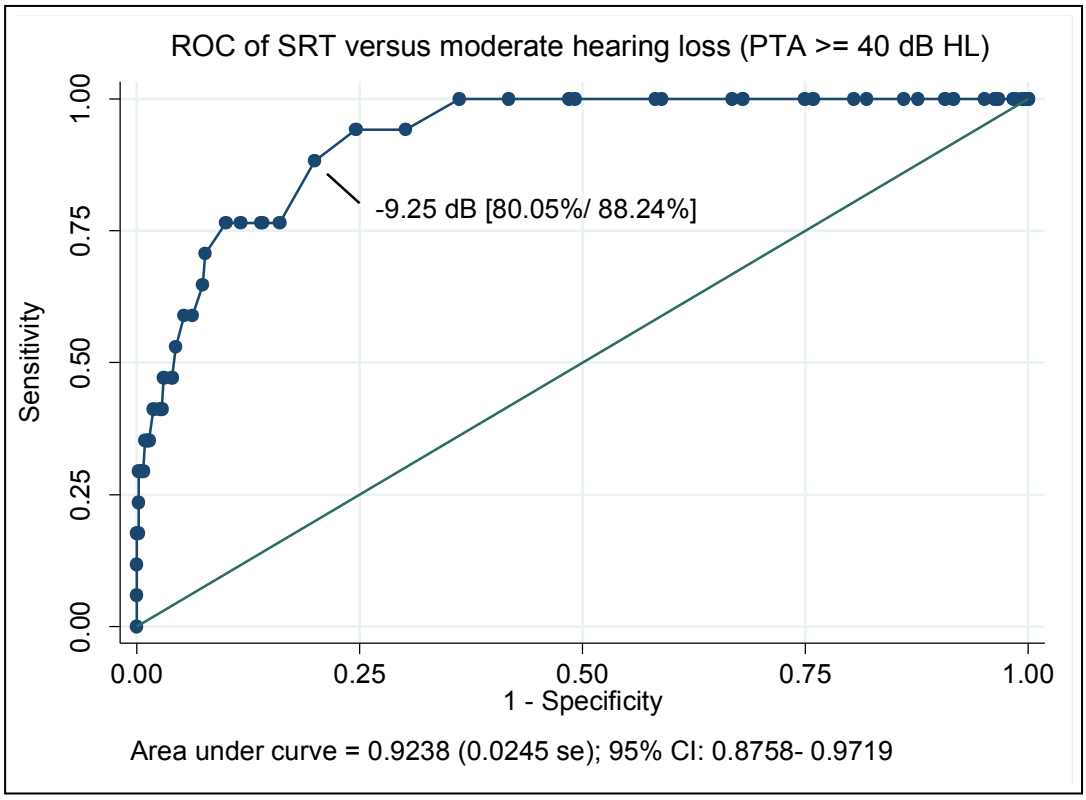


Figure 19 Receiver operating curve of SRTs against moderate hearing loss

The receiver operating curve (ROC) plots the specificity versus the sensitivity of the speech reception threshold (SRT) in comparison to hearing loss as measured by the pure-tone average ≥ 40 dB HL. At a SRT of -9.25 dB, a sensitivity and specificity of 88.24% and 80.05% was observed, respectively. The area under the curve is presented with standard error (se) and 95% confidence intervals (95% CI).

Bivariate heritability analysis of speech reception in noise and pure-tone audiometry

The strongest correlation in heritability estimates was observed for transformed SRT and PTA values ($r(a^2)$= -0.7 (95% CI:-1.0- -0.5)). However, the strength of correlation decreased after age-adjustment of both SRT and PTA values to $r(a^2)$= -0.4 (95% CI:-1.0-1.0), which was also reflected in wider confidence intervals. The correlation between unique environmental factors for both tests was generally lower than correlation within heritability estimates ($r(e^2)$= -0.3 (95% CI: -0.5 - -0.1) to $r(e^2)$= -0.4 (95% CI:-0.5- -0.1). Although univariate heritability estimates for the SRT were low under the AE model (A_{SRT}=15% - 27%), the correlation between heritability estimates for SRT and PTA values was consistent, supporting the existence of shared genetic factors between both hearing phenotypes. The percentage of phenotypic correlation between the corresponding SRTs and PTA values due to shared additive genetics (Equation 16) was estimated as 68.8% and 36.8% for transformed values and age-adjusted residuals, respectively.

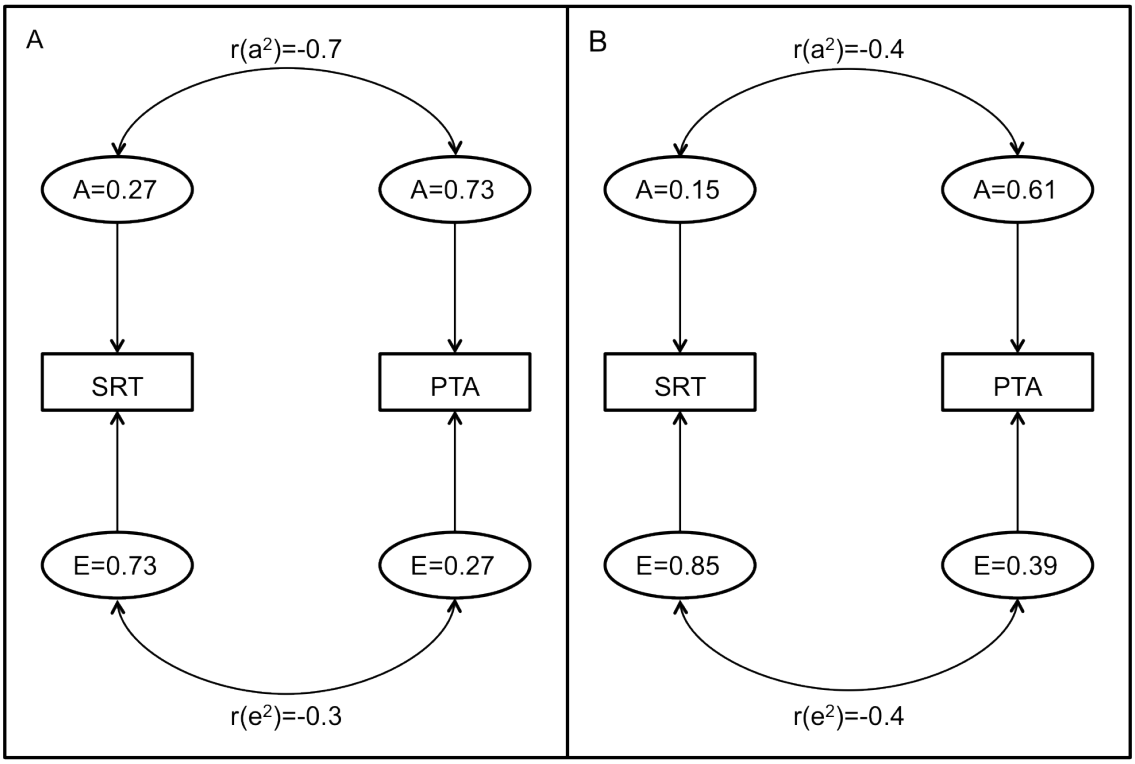


Figure 20 Path diagrams of bivariate heritability estimates for SRT and PTA values

The path diagrams depict the results of the bivariate heritability analysis between SRT and PTA values based on the AE model. Observed phenotypes and latent variables are indicated by black boxes and circles, respectively. Estimated variances explained by additive genetics (A) and unshared environment (E) are given for each phenotype (SRT or PTA). Correlation coefficients between additive genetic factors ($r(a^2)$) and unique environmental factors ($r(e^2)$) are indicated by double-headed arrows. The effect of the latent variables on the respective phenotype is indicated by single headed arrows. Panel A displays the results of the bivariate heritability analysis of transformed SRT and PTA values, while panel B represents results for age-adjusted SRT and PTA residuals.

Table 26 Results of the bivariate heritability analysis of SRT and PTA measures.

phenotype	model fit			model comparison				univariate estimates % (95% CI)	correlation between estimates (95% CI)
	model	-2 log L	df	Δ -2 log L	Δ df	p-value	AIC	A C E	$r(a^2)$ $r(c^2)$ $r(e^2)$
transformed SRT & PTA	ACE	-7535.734	885	-	-	-	-	18 (0-.44) 8 (0-32) 74 (56- 91) 46 (9- 79) 27 (0-58) 27 (20- 39)	-1.0 (-1.0 -1.0) -0.2 (-1.0-0.7) -0.3 (-0.5 --0.1)
	AE	-7532.57	888	3.17	3	0.37	-2.83	27 (7-45) - 73 (55-93) 73 (63- 81) - 27 (19-37)	-0.7 (-1.0- -0.5) - -0.3 (-0.5 - -0.1)
age-adjusted SRT & age-adjusted PTA	ACE	-7850.84	885	-	-	-	-	8 (0- 32) 6 (0-23) 85 (67- 99) 53 (13- 72) 8 (0-42) 39 (27- 56)	-1.0 (-1.0- 1.0) 1.0 (-1.0- 1.0) -0.3 (-0.5- -0.1)
	AE	-7849.14	888	1.70	3	0.64	-4.30	15 (0-35) - 85 (65- 100) 61 (45- 73) - 39 (27- 55)	-0.4 (-1.0-1.0) - -0.4 (-0.5- -0.1)

In the bivariate structural equation modelling an ACE model (Model) was fitted to the observed phenotypic variance in both SRT and PTA measures and correlation between univariate variance components for both traits were determined. Model fit was based on maximum likelihood estimation. For each trait, the nested AE model was compared to the full ACE model, taking into account additive genetics (A) and unshared environmental factors (E), Model fit for the full and respective reduced model were compared in a likelihood ratio test (Δ -2 log L). Significance of the likelihood ratio test (p-value) is based on a chi-square statistic with 1 or 2 degrees of freedom (Δdf). In addition, the Akaike's information criterion (AIC) gives a measure of parsimony of a reduced model in comparison to the full model. For each phenotype the ACE model fit and nested models with a better model fit (highlighted in grey) are shown. Model comparison is only given for nested models as they are compared to the full (ACE) model. Estimated variances explained by the specific causal factors (A= additive genetics, C= shared environment and E= unshared environment) are given with 95 % confidence intervals (95% CI) for each model. Correlation coefficients between additive genetic factors ($r(a^2)$), shared environmental factors ($r(c^2)$) and unique environmental factors ($r(e^2)$) are given with 95% confidence intervals.

Discussion

In this chapter a web-based speech-perception test was compared to hearing ability as measured by pure-tone audiometry in respect to future genetic studies. Speech reception in noise was primarily influenced by additive genetic factors and environmental exposure unshared within twin pairs. Correlation between hearing ability as measured by SRT and PTA was explained by up to 68.8% by additive genetic factors shared between both hearing traits. In addition, speech reception in noise showed 88.24% sensitivity and 80.05% specificity to correctly identify subjects with moderate hearing loss as defined by a PTA≥ 40 dB HL.

SRTs in the TwinsUK sample were collected using a web-based speech-in-noise test. This test has been developed by Action on Hearing Loss based on the digits-in-noise test by *Smits et al.*[13]. In comparison to previous speech-in-noise tests, it uses triple digits (i.e. 3-7-5) as speech material rather than complete sentences [8] to reduce the effect of cognitive ability on word recognition. Furthermore the speech signal in this test is presented against increasing background noise, to mimic real life situations. It should also be mentioned that in the test applied here, speech intensity is kept constant while the noise component varies, whereas in the original Dutch version [13] speech volume was allowed to increase or decrease against a constant background noise.

In prospect of future genetic studies, the univariate heritability of different speech-in-noise phenotypes was determined. For all three SRT phenotypes analysed, the AE model was most likely under the observed trait variance. This implies that shared environment within twin pairs does not have a major impact on speech reception in noise. The same effect has been determined in chapter 3 for different pure-tone audiogram measures. Unique environmental factors explained E=66% (95% CI: 58-75) to E=81% (95% CI: 72-92) of variance in SRTs, while additive genetic factors accounted for the remaining A=34% (95% CI: 26- 42) to A=20% (95% CI: 10- 29). This is contradictory to hearing as measured by pure-tone audiometry, where genetic factors explained more than half of the variance in hearing (chapter 3). Heritability of speech intelligibility has been determined in elderly women of the Finnish Twin Study on Aging. Speech reception was measured as the better ear speech recognition threshold (BESRL). Variance in BESRL values was determined to 66% (95% CI: 55-74) by additive genetic factors [29], twice as high as our estimates, however, this test measured speech recognition in a quiet environment [29]. *Smoorenburg* [30] measured speech perception in 200 individuals with noise induced hearing loss. He observed only moderate correlation (r=0.45) between SRTs in noise and quiet environments,

indicating that environmental exposure (i.e. noise exposure) might influence speech reception in noise and quiet differently. In addition, tone perception thresholds at 2 and 4 kHz were determined as a good predictor for SRTs in noise (r=0.72) [30]. The low but consistent heritability of SRTs merits further analyses in genetic studies, however, a high sample size will be required to achieve sufficient statistical power to identify genetic variants associated with speech reception in noise.

In a purely female sample (n=1684), univariate heritability estimates for STR values were slightly decreased (A=19%, 95% CI: 8- 28) in comparison to the mixed gender estimates (A=20%, 95% CI: 10-29). It would have been interesting to compare univariate heritability estimates of speech reception in noise between genders. However, the number of complete twin pairs for the male participants (n=49 pairs) was too low to achieve sufficient statistical power to use structural equation modelling. Previous power calculations for univariate heritability estimation report that at least 200 twin pairs are required to achieve sufficient statistical power [31].

To explore the relationship between speech reception in noise measures and pure-tone audiometry, correlation between different speech reception in noise and pure-tone audiogram phenotypes was investigated. Moderate correlations could be observed between pure-tone averages and SRTs (r=0.52--0.32). *Smits et al.* [13] observed an even stronger correlation between speech in noise test results for telephone usage and PTAs for medium (0.5-2 kHz) and medium to high (0.5-4kHz) frequencies (r=0.732 and r=0.770, respectively). The speech-in-noise test for telephone usage [13] has previously been validated against an existing speech-in-noise hearing test using sentences rather than triple digits as speech material [8]. Furthermore, it has been regressed against pure-tone averages for different frequency ranges (0.5-4 kHz) [13]. Most audiologists consider the air conduction pure-tone audiogram as the standard hearing test. Although we were aware that speech reception and particular speech reception in background noise might be determined by other factors than pitch perception alone, we wanted to measure the sensitivity and specificity of the speech in noise test as a predictor for moderate hearing loss as determined by pure-tone audiometry. A PTA ≥ 40dB HL is considered as definition for moderate hearing loss [28] and was thus applied as a reference measurement for hearing loss. Sensitivity and specificity at an SRT of -9.25 dB was high in comparison to the PTA (88.24% and 80.05%, respectively).

These sensitivity and specificity values might be too low for a diagnostic test, but might serve as a good predictor of overall hearing, as used here. Difficulty to understand speech against background noise is a symptom of hearing loss that may have different sources. Speech perception is defined both by the anatomy and physiology of the ear as well as sound processing in the brain. The speech-in-noise test cannot discriminate the source of the hearing loss. In comparison, air and bone conduction pure-tone audiometry can be used to differentiate between conductive, sensorineural or mixed hearing loss and thus help to identify affected structures in the ear. Even though the speech-in-noise test showed high sensitivity (88.24%) and specificity (80.05%) to correctly identify individuals with moderate hearing loss, further tests would be required to follow up the type of hearing loss. The data presented here suggests that the web-based speech-in-noise test could be used to screen for hearing loss in the general population. Individuals identified with hearing loss should then be referred to an audiologist for further diagnosis.

The moderate sensitivity and specificity of the speech-in-noise test in comparison to pure-tone audiometry lead to the hypothesis that tests measuring similar hearing traits might also share a proportion of heritability. Therefore a bivariate heritability analysis was conducted, which estimated the univariate variance components for both traits and the correlation between the corresponding components. Genetic correlations between pure-tone averages and SRT values determined in the bivariate analyses ranged from r = −0.7 to r = −0.4 and explained 68.8% and 36.8% of the phenotypic correlation between both measures of hearing ability. In a female Finnish twin sample it was shown that better ear hearing threshold levels (measured using pure-tone audiometry) and BESRL values (speech reception in quiet) had a genetic component in common, which accounted for 54% (95%CI: 43-64) of the variance in speech recognition [29]. Although bivariate heritability for speech reception and pitch perception in the Finnish Twin cohort was based on different measures of hearing ability and fitted to a different bivariate variance component model, these results support our findings of shared heritability between hearing ability as measured by speech perception and pure-tone audiometry.

These results suggest that part of the variance in both SRTs and PTAs is determined by additive genetic factors shared between both hearing measures. Genetic studies aimed at determining genetic variants associated with hearing ability could therefore use hearing ability as measured by either of these two hearing test (speech-in-noise test and pure-tone audiometry) and thereby increase the sample size and statistical

power of future genetic studies. Alternatively, the shared heritability and moderate specificity and sensitivity estimates support the use of samples with SRT data as validation samples for future genetic studies of hearing ability as measured by pure-tone audiometry. According to the sensitivity and specificity as well as the moderate bivariate heritability correlations determined, it was concluded that the speech-in-noise test represents a satisfactory predictor for hearing loss as measured by the pure-tone audiogram. Although the SRT test was slightly less sensitive and specific in identifying HL correctly compared to pure-tone audiometry, much larger sample sizes could be recruited at minimal time and effort.

There were limitations, which were beyond the scope of this study. The number of unpaired twins was relatively high for data collected from a twin cohort. We explain this high number by the recruitment and data collection procedure. Whereas pure-tone audiograms were performed at St. Thomas hospital, during a twin visit including both twins of a pair, the web-based speech-in-noise test was performed at the volunteer's home, separate of their twin sibling. Furthermore, the number of individuals with both speech reception in noise measures and pure-tone audiometry was limited (n=448). Nevertheless, hearing ability in this sample reflected the shape of the hearing distribution in the complete speech-in-noise test and pure-tone audiogram samples. As reported for the complete pure-tone audiogram sample in chapter 2, hearing ability was better than reported for other European samples of this age range previously [32]. The number of individuals with moderate hearing loss was thus limited in the speech-in-noise test validation analysis. We also accept that speech reception in noise and pitch perception at different frequencies in quiet represent different aspects of hearing function and the comparison of both should therefore be interpreted with caution. Finally, ambient noise levels during the web based hearing test could not be controlled for. All volunteers were asked to perform the test in a quiet environment trying to avoid interruption. However, *Smits et al* has shown that ambient noise levels and quality of PC speakers should only have limited affect on the resulting speech-to-noise ratio [13].

In conclusion, this is the first study to our knowledge that explores the heritability of SRTs (measured via the speech-in-noise test) and validates this measure of hearing ability against standard pure-tone audiometry. These results suggest, that speech reception in noise shows a high sensitivity and moderate shared heritability with hearing ability as measured by pure-tone audiometry and could thus be used as a predictor for moderate hearing loss as measured by pure-tone audiometry in future

studies. The web-based set-up of this test allows collection of large datasets for future epidemiological studies at reduced time and effort compared to pure-tone audiometry.

References

1. Dalton DS, Cruickshanks KJ, Klein BE, Klein R, Wiley TL, et al. (2003) The impact of hearing loss on quality of life in older adults. Gerontologist 43: 661-668.
2. (2007) Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature 447: 661-678.
3. Alpay LL, Toussaint PJ, Ezendam NP, Rövekamp TA, Graafmans WC, et al. (2004) Easing Internet access of health information for elderly users. Health Informatics Journal 10: 185-194.
4. Campbell RJ, Nolfi DA (2005) Teaching elderly adults to use the Internet to access health care information: before-after study. Journal of medical Internet research 7.
5. Erlanger DM, Kaushik T, Broshek D, Freeman J, Feldman D, et al. (2002) Development and Validation of a Web-Based Screening Tool for Monitoring Cognitive Status. The Journal of Head Trauma Rehabilitation 17: 458-476.
6. Farvolden P, McBride C, Bagby RM, Ravitz P (2003) A web-based screening instrument for depression and anxiety disorders in primary care. Journal of medical Internet research 5.
7. Boothroyd A (1968) Developments in speech audiometry. British Journal of Audiology 2: 3-10.
8. Plomp R, Mimpen A (1979) Improving the reliability of testing the speech reception threshold for sentences. International Journal of Audiology 18: 43-52.
9. Nilsson M, Soli SD, Sullivan JA (1994) Development of the Hearing In Noise Test for the measurement of speech reception thresholds in quiet and in noise. The Journal of the Acoustical Society of America 95: 1085-1099.
10. Wilson RH, Burks CA (2005) Use of 35 words for evaluation of hearing loss in signal-to-babble ratio: A clinic protocol. Journal of rehabilitation research and development 42: 839.
11. Causey GD, Hood LJ, Hermanson CL, Bowling LS (1984) The Maryland CNC test: normative studies. International Journal of Audiology 23: 552-568.
12. -3. I (1996) Acoustics: Audiometric test methods part 3: Speech audiometry. International Organization for Standardization.

13. Smits C, Kapteyn TS, Houtgast T (2004) Development and validation of an automatic speech-in-noise screening test by telephone. International Journal of Audiology 43: 15-28.

14. Smits C, Houtgast T (2006) Measurements and calculations on the simple up-down adaptive procedure for speech-in-noise tests. The Journal of the Acoustical Society of America 120: 1608.

15. Smits C, Kramer SE, Houtgast T (2006) Speech reception thresholds in noise and self-reported hearing disability in a general adult population. Ear and Hearing 27: 538-549.

16. Smits C, Merkus P, Houtgast T (2006) How we do it: The Dutch functional hearing–screening tests by telephone and internet. Clinical Otolaryngology 31: 436-440.

17. Jansen S, Luts H, Wagener KC, Frachet B, Wouters J (2010) The French digit triplet test: A hearing screening tool for speech intelligibility in noise. International Journal of Audiology 49: 378-387.

18. Ozimek E, Kutzner D, Sęk A, Wicher A (2009) Development and evaluation of Polish digit triplet test for auditory screening. Speech Communication 51: 307-316.

19. Smits C, Goverts ST, Festen JM (2013) The digits-in-noise test: Assessing auditory speech recognition abilities in noise. The Journal of the Acoustical Society of America 133: 1693-1706.

20. ActionOnHearingLoss (2011) Check your Hearing- online hearing test. London: Action on Hearing Loss.

21. Lutman ME, Hall SJ, Athalye S (2006) Development of a telephone hearing test.

22. Neale MC, Cardon LR, North Atlantic Treaty Organization. Scientific Affairs Division. (1992) Methodology for genetic studies of twins and families. Dordrecht ; Boston: Kluwer Academic Publishers. xxv, 496 p. p.

23. Falconer DS (1981) Introduction to quantitative genetics: Longman.

24. Akobeng AK (2007) Understanding diagnostic tests 1: sensitivity, specificity and predictive values. Acta Pædiatrica 96: 338-341.

25. Loehlin JC (1996) The Cholesky approach: A cautionary note. Behavior Genetics 26: 65-69.

26. Rijsdijk FV, Sham PC (2002) Analytic approaches to twin data using structural equation models. Briefings in Bioinformatics 3: 119-133.

27. Snieder H (2000) Path analysis of age-related disease traits. In: Spector TD, Snieder H, MacGregor AJ, editors. Advances in twin and sib-pair analysis. London

Oxford ; New York: Greenwich Medical Media ;

Distributed worldwide by Oxford University Press. pp. xv, 266 p.

28. World Health Organisation (2011) Grades of hearing impairment. In: Organization WH, editor. Prevention of Blindness and Deafness Geneva: World Health Organization.

29. Viljanen A, Era P, Kaprio J, Pyykkö I, Koskenvuo M, et al. (2007) Genetic and Environmental Influences on Hearing in Older Women. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences 62: 447-452.

30. Smoorenburg GF (1992) Speech reception in quiet and in noisy conditions by individuals with noise‐induced hearing loss in relation to their tone audiogram. The Journal of the Acoustical Society of America 91: 421-437.

31. Martin NG, Eaves LJ, Kearsey MJ, Davies P (1978) The power of the classical twin study. Heredity 40: 97-116.

32. Cruickshanks KJ, Wiley TL, Tweed TS, Klein BE, Klein R, et al. (1998) Prevalence of hearing loss in older adults in Beaver Dam, Wisconsin. The Epidemiology of Hearing Loss Study. Am J Epidemiol 148: 879-886.

Chapter 5: Genome-wide association studies of hearing ability with age

Abstract

Heritability estimates determined in chapter 3 and 4 confirmed a moderate heritability for hearing ability with age in TwinsUK, proofing this sample suitable for genetic studies. This chapter aims to determine genetic variants associated with hearing ability with age in a predominantly female sample.

Genetic variants associated with ARHI and hearing function were sought in a genome-wide association design using three phenotypes of hearing ability with age (age-adjusted PCs (PC1; PC2) and PTA residuals calculated from pure-tone audiograms) and a measure of hearing function (age-and gender adjusted SRT residuals). Genome-wide association was performed on a marker and gene-based level and replication sought in two validation datasets. Significantly associated ($p<10^{-3}$) genes were followed up in literature and tested for gene enrichment.

Despite a moderate sample size (n=1028-1214), no genome-wide significant associations ($p\leq5x10^{-8}$) with hearing ability could be determined. Nevertheless, suggestive genome-wide significant associations ($p<5x10^{-6}$) were identified for genes *LAMA2*, *GLRX3*, *SDK1*, *HSPG2*, *FGF14* and *XKR3*. In the gene-based analysis *FAM110C*, *GLRX3* and *DHRS7C* presented the most highly associated genes ($p<10^{-4}$). Gene-based validation was determined for genes *PANX1*, *LYST* and *WNT16*. In the gene enrichment analysis associated genes were enriched for ephrin receptors, genes involved in cell-cell interaction and gene silencing by miRNA.

In conclusion, this analysis determined suggestive significant associations with various genes with a putative function in hearing ability. The lack of genome-wide significant associations might be explained by the polygenic nature of ARHI, thus reducing the effect size of single variants. Future GWAS of ARHI should focus on larger sample sizes to increase the power to detect genome-wide significant associations with common genetic variants of low to moderate effect sizes.

Introduction

Heritability studies attempt to distinguish the proportion of phenotypic variance determined by environmental or genetic factors. Heritability estimates for hearing ability with age in the TwinsUK cohort ranged from 39% (speech reception in noise, chapter 4) to 77% (PC1 in older samples; chapter 3), confirming a moderate genetic effect and suitability of this sample for future genetic studies. Genetic variations causing differences in quantitative phenotypes (i.e. blood pressure, height, hearing ability) are referred to as quantitative trait loci. Detection of these loci has been a major aim for geneticists in the last decade. In case of ARHI, candidate gene, linkage and association studies have been used to define genetic variants associated with this trait.

Linkage Analyses

Linkage analysis are based on the hypothesis that markers located close to a disease allele are linked to this allele and that segregation of these markers is related to the inheritance of disease in large pedigrees. Linkage disequilibrium describes the phenomenon that some alleles are inherited together more often than expected by chance. These markers are usually located close to each other on a chromosome and therefore less likely to be separated by recombination during meiosis. Linkage analysis makes use of linkage disequilibrium by selecting markers that can easily be traced from generation to generation. Nevertheless, large pedigrees are required to narrow down linkage intervals, as family members share a higher proportion of alleles identical by decent than unrelated individuals. Collection of large pedigrees is particularly difficult in the study of age-related traits due to the late age of disease onset. Even with large pedigrees available, success is often limited due to multiple loci implicated in disease aetiology and large linkage intervals, which might include several possible candidate genes.

Genetic markers used in most studies include copy number variations and single nucleotide polymorphisms (SNPs). SNPs represent the most common genetic variation, occurring roughly every 1000 base pairs per human genome. Most SNPs are located outside protein coding regions and have therefore no direct impact on the protein sequence. Only a minor proportion of SNPs is located in coding regions and therefore has a putative impact on the protein.

The first linkage analysis of ARHI was performed in 328 families (1789 subjects) of the Framingham cohort [1]. Pure tone averages calculated for the lower (0.25, 0.5 and 1.0 kHz) and medium (0.5, 1.0 and 2.0 kHz) frequencies accounted for the quantitative

phenotype. Analysis resulted in 6 loci with logarithm of the odds (LOD) scores > 1.5, on chromosomes 11, 10, 14 and 18.

Garringer et al [2] used a sibling–pair linkage analysis to detect genetic loci linked to ARHI. This method aims to identify genetic markers shared identical by descent to a higher percentage than expected for DZ twins. Linkage analysis resulted in a region of 23 cM on chromosome 3 (between markers D3S2496 and D3S3637) with a LOD score of 2.5. The D3S1292 marker included in this region maps to the *DFNA18* locus (autosomal dominant hearing loss) [3].

A third linkage analysis was conducted on 200 European and Finnish sibships (1081 subjects) using PCs (PC1-3) calculated from pure-tone thresholds. After simulation, a region on chromosome 8 achieved genome-wide significance (p=0.0170) in the multipoint analysis. The region on chromosome 8 mapped to locus 8q24.13-q24.22[4].

Candidate gene studies

Another approach to determine QTLs for common diseases involves sequencing of candidate genes. However, an a priori hypothesis is required to select biological candidates, which is particularly challenging in a poorly understood trait like ARHI. In addition, sequencing of selected candidate genes in several cases and controls is a time and cost intensive process. Furthermore, the candidate gene method assumes that mutations in a single gene could have a sufficient effect on a trait to be determined in this study design, which might not apply to a polygenic trait with many variants of low effect sizes on the trait [5].

Unal et al [6] measured the association of 4 different N-acetyltransferase polymorphisms with susceptibility to ARHI. The enzyme encoded by *NAT2* plays an important role in acetylation of drugs and environmental toxins, leading to removal of the latter. Polymorphisms in *NAT2* have been shown to influence enzyme activity [7]. The study sample included 68 subjects with ARHI and 98 healthy controls. Multivariate logistic regression analysis was used to determine the association between N-acetyltransferase 2 (*NAT2)* polymorphism genotypes and hearing status. Subjects homozygous mutant for *NAT2*6A* (G590A) showed a 15.2 fold (p=0.013) increased risk of developing ARHI compared to individuals with a homozygous wildtype genotype. Individuals heterozygous at this locus showed a 0.34-fold (p=0.032) decreased risk of ARHI compared to the homozygous mutant group.

These results motivated *Van Eyken et al* [8] to conduct a study on the effect of polymorphisms in genes associated with reactive oxygen species detoxification and ARHI. Selected genes of interest were the previously reported *NAT2* and different classes of glutathione S-transferases. Individuals from 9 centres across Europe were tested for hearing levels and grouped according to a Z-score for the high and low frequencies. All samples were checked for population stratification and according to that, separated into a Finnish sample group and a general European group. Two significant associations between genotypes and phenotypes could be identified in the Finnish population. An association between Z high and homogeneity for the *GSTT1* polymorphism in women (p=0.035) and an association between the *GSTM1* wildtype and Z high (p= 0.027) was determined. Analysis in the European group showed a significant association between Z high and *NAT2*6A* (for the AA genotype, p= 0.013).

Van Laer et al [9] conducted an association study on 70 candidate genes for ARHI. 2318 subjects from 9 centres across Europe were genotyped for 703 SNPs located across the candidate genes and regions 3000 bp upstream of these genes. The three top ranked SNPs in the results were located at the grainyhead like 2 (*GRHL2*) locus on chromosome 8q22.3 and were in LD with one another. Odds ratios (ORs) for the top SNP (rs10955255) ranked between 1.76 and 1.02 at the different centres and, consistent with this, the two top ranked SNPs showed ORs following the same direction. Fine mapping resulted in the discovery of a third significant intronic SNP (rs13263539, p=0.0002) in *GRHL2* associated with ARHI. *GRHL2* encodes a transcription factor expressed in cells lining the cochlear duct.

Genome-wide association studies

Novel DNA microarrays capture about 1 million SNPs and copy number variations. These SNPs have been selected as special tagging or marker SNPs, which are in strong LD with surrounding variants, giving maximal haplotype information according to the HapMap haplotype database [10]. Imputation based on these tagging SNPs and corresponding haplotypes can yield coverage of up to several million SNPs per subject. Genome-wide association studies (GWAs) test the association of these SNPs with a phenotype. It is assumed that significantly associated marker SNPs will be in close LD and/or on the same haplotype block as the causal genetic variant. GWAs studies are based on the common disease common variant hypothesis [11,12], which assumes that disease causing alleles are equally common in the population as the trait itself. Tagging SNPs in linkage equilibrium with the causative allele, or even the causative allele itself, are expected to be significantly more prevalent in individuals sharing a certain phenotype than in individuals of a different phenotype. This method requires no

hypothesis regarding diseases pathways and was designed for unrelated subjects rather than large pedigrees. Putative relation between participants has to be controlled and adjusted for [13].

To date, three GWASs of ARHI have been published. The first analysis was conducted as a combination of linkage study and GWAS on 1081 individuals [4]. No significant association signals were identified, but linkage resulted in significant linkage peaks on chromosome 8q [4].

The second GWAS for ARHI was performed on 846 cases and 846 controls for ARHI using a pooling approach [14]. A set of 23 top ranked SNPs was used for replication in a group including 63 cases and 67 controls. The T-allele of rs11928865 was associated with ARHI in the original European and the replication European samples ($p=9x10^{-5}$). This SNP was located in the metabotropic glutamate receptor type 7 (*GRM7)* gene. It could be shown, that GluR7, the protein product of *GRM7,* is expressed in the sensory epithelium of the organ of Corti, in the hair cells of the vestibular apparatus and in the spiral ganglion of mice at different developmental stages. In addition, expression could be detected in the inner ear of a human adult, underlining the involvement of GluR7 in hearing ability.

A third GWAS on ARHI has been performed in the Finnish Saami [15]. The high extent of linkage disequilibrium in this isolated population offers a powerful advantage over outbred populations, as fewer tagging SNPs will be required to obtain full haplotype information. Nevertheless, isolated populations might be distinct in their genetic and environmental factors, making it difficult to apply findings obtained in these groups to the general population. Association testing was performed in 347 subjects using three PCs (PC1, PC2, PC3) as quantitative phenotypic traits obtained from the pure-tone audiogram. One SNP (rs457717) associated with PC3 reached suggestive genome-wide significance levels ($p=3.55x10^{-7}$). The two top ranked SNPs for PC3 mapped to the IQ-motif containing GTPase activating like protein (*IQGAP2*). *IQGAP2* is important for signalling pathway regulation and shows expression inside the cochlea [15]. It has also been associated with cadherins, which are involved in sensory hair cell viability [15].

A very recent GWAS was performed in the ARHI cohort by *Fransen et al.* [16]. This study was the first to use imputation based on data from the 1000 Genomes panel, resulting in <4000000 markers tested for association with PC1, PC2 and PC3 in 1489 samples. Subjects for this analysis were recruited as a population-based sample from

residential areas surrounding Antwerp, Belgium. After principal component analysis based on pure-tone thresholds, only samples with PC values within the highest or lowest 20% percentiles were selected for analysis, resulting in 1489 subjects. Initial power calculations based on the described study design predicted 80% power to detect genome-wide significant associations with genetic variants explaining 2% of trait variance, while twice the sample size would be required to identify genes accounting for 1% of phenotypic variance. GWAS analysis failed to determine genome-wide significant associations with any of the three PC phenotypes. Furthermore, replication of previously reported genome-wide associations [14,15,17] were scarce. Even after adjustment for environmental exposure and taking into account rare genetic variants, no genome-wide significant associations could be determined [16]. A genome-wide complex trait analysis in this sample estimated that 22% of phenotypic variance could be explained by the collective effect of all SNPs in the sample. *Fransen et al.* conclude that ARHI is likely to be caused by multiple genetic variants each with low effect on the trait[16].

Table 27 Summary of recent candidate gene, linkage and genome-wide association studies of ARHI

study design	reference	phenotype	n	age [years]	loci / gene [SNP]	p-value / LOD score
candidate gene	Unal *et al.* 2005	PTT,	68 cases, 98 controls	cases: 61±6.46 controls: 60.3±3.04	NAT2*6A [G590A]	p=0.013
	Van Eyken *et al.* 2007b	Z-scores	530	53-67	*GSTT1*	p=0.035
					GSTM1 wildtype	p=0.027
			2010	53-67	*NAT2*6A* [AA genotype]	p=0.013
	Van Laer *et al.* 2008	Z-scores	2318	53-67	*GRHL2* [rs10955255]	p=8.38x 10^{-5}
linkage analysis	DeStefano *et al.* 2003	PTA	328 families (n=1789)	32-89	chr 11 (2,79 and 143 cM); chr 10 (171 cM); chr 14 (126 cM); chr 18 (116 cM)	LOD>1.5
	Garringer et al. 2006	Self reported hearing loss	160 DZ twin pairs	69-82	DFNA18 [D3S1558]	LOD=2.5
	Huyghe *et al.* 2008	PCs	200 sibships (n=955)	49-76	8q24.13-q24.22 [rs4512366]	p=0.0170 LOD =4.23
GWAS	Huyghe *et al.* 2008	PCs	955	49-76	-	-
	Friedman *et al.* 2009	Z-scores	846 cases and 846 controls	53-67	*GRM7* [rs11928865]	p=9x10^{-5}
	Van Laer *et al.* 2010	PCs	347	50-75	*IQGAP2* [rs457717]	p=3.55x 10^{-7}
	Fransen *et al.* 2014	PCs	1489	-	no genome-wide significant associations found	-

Summary of recent candidate gene association, linkage and genome-wide association studies (GWAS) of ARHI. Five different phenotypes were defined: self reported hearing loss, pure-tone thresholds (PTT), Z-scores, pure-tone averages (PTA) or principal components (PCs) calculated from the audiogram. In case of Z-scores, the 34% extreme subjects of all samples (n) were chosen as cases and controls. Sample age is given as age range or mean age ± standard deviation. Significance of linkage or association of a specific locus or polymorphism with ARHI is given in p-values or logarithm of the odds (LOD) scores.

Complex diseases are often caused by interplay of genetic risk genotypes and exposure to environmental risk factors. Several environmental risk factors have been proposed for ARHI, whereas suggested genetic risk loci remain to be replicated. The research by *Fransen et al.* supports a polygenic character for ARHI [16].

In chapter 3, hearing ability with age showed a moderate heritability in females of the TwinsUK cohort. This suggests additive genetic factors involved in the pathology of this common trait. Accordingly, the aim of this chapter was to identify genetic variants for hearing ability with age in GWAs using the SRT values and PCs and PTAs calculated from the pure-tone audiogram data.

Materials and Methods

Genotyping

The Wellcome Trust Sanger Institute performed genotyping, genotyping data QC and merge of data, as well as imputation. Genotyping of the TwinsUK dataset was done with a combination of Illumina arrays (HumanHap300 [18,19], HumanHap610Q, 1M-Duo and 1.2MDuo 1M). The normalised intensity data was pooled [20] for each of the three arrays separately (with 1M-Duo and 1.2MDuo 1M pooled together). For each dataset the Illluminus calling algorithm [21] was used to assign genotypes in the pooled data. No calls were assigned if an individual's most likely genotype was called with less than a posterior probability threshold of 0.95. Validation of pooling was achieved via a visual inspection of 100 random, shared SNPs for overt batch effects. Finally, intensity cluster plots of significant SNPs were visually inspected for overdispersion biased no calling, and/or erroneous genotype assignment. SNPs exhibiting any of these characteristics were discarded.

Genotyping data QC

Similar exclusion criteria were applied to each of the three datasets separately. *Samples*: Exclusion criteria were: (i) sample call rate <98%, (ii) heterozygosity across all SNPs ≥2 s.d. from the sample mean; (iii) evidence of non-European ancestry as assessed by PCA comparison with HapMap3 populations; (iv) observed pairwise IBD probabilities suggestive of sample identity errors; (v). Misclassified monozygotic and dizygotic twins were corrected based on IBD probabilities. *SNPs:* Exclusion criteria were (i) Hardy-Weinberg p-value<10^{-6}, assessed in a set of unrelated samples; (ii) MAF<1%, assessed in a set of unrelated samples; (iii) SNP call rate <97% (SNPs with MAF≥5%) or < 99% (for 1% ≤ MAF < 5%). Alleles of all three datasets were aligned to HapMap2 or HapMap3 forward strand alleles.

Data merge

Prior to merging, pairwise comparison among the three datasets and further excluded SNPs and samples was performed to avoid spurious genotyping effects, indentified as follows: (i) concordance at duplicate samples <1%; (ii) concordance at duplicate SNPs <1%; (iii) visual inspection of QQ plots for logistic regression applied to all pairwise dataset comparisons; (iv) Hardy-Weinberg p-value<10^{-6}, assessed in a set of unrelated samples; (v) observed pairwise IBD probabilities suggestive of sample identity errors. The three datasets were then merged, keeping individuals typed at the largest number of SNPs when an individual was typed at two different arrays. The merged dataset consists of 5,654 individuals (2,040 from the HumanHap300, 3,461 from the HumanHap610Q and 153 from the HumanHap1M and 1.M arrays) and up to 874,733 SNPs depending on the dataset (HumanHap300: 303,940, HumanHap610Q: 553,487, HumanHap1M and 1.M: 874,733).

Imputation

Imputation was performed using the IMPUTE software package (v2) [22] using two reference panels, P0 (HapMap2, rel 22, combined CEU+YRI+ASN panels) and P1 (610k+, including the combined HumanHap610k and 1M reduced to 610k SNP content).

Selection of participants

Participants for the hearing ability with age GWAs were chosen according to two criteria: Firstly, availability of phenotype results (pure-tone audiogram or speech-in-noise test) and secondly availability of corresponding genotyping data. In dizygotic twin pairs, both siblings were included in the analysis if genotyping and phenotype data were available for both siblings. In case of monozygotic twin pairs, who are expected to share 100% of alleles, primarily one sibling had been genotyped previously, to reduce the number of genotyping duplicates. Which MZ twin sibling of a pair was genotyped had been selected previous to the start of this study and was therefore not biased by the selection of better or worse hearing twins. Twins whose co-twin had missing genotyping or phenotype data were treated as singletons. To adjust for the increased relatedness within complete twin pairs, a polygenic model [23] was applied as part of the association study, as described below. Genotyping had been performed for 1028 and 1214 individuals with pure-tone audiogram and speech-in-noise data, respectively. Demographic and phenotypic characteristics of these individuals are presented in Table 28.

Association analysis

Genome-wide association analysis was performed for 5 separate traits: age-adjusted PTA residuals and unadjusted PTAs, age-adjusted PC1 and PC2 residuals as well as normalized age and gender adjusted SRT residuals. GenABEL R library was used [24] for the association analysis. The analysis was performed in a two step mixed model and regression analysis [23,25]. In the first step a mixed model was applied to the trait (i.e. PC1, PC2, SRT) with optional environmental covariates (i.e. age, gender) as fixed effects and a kinship matrix calculated from genomic data as random effect. Fitting of the mixed model was based on a maximum likelihood estimate and performed using the polygenic() function by Aulchenko and Svischeva [23,25]. Objects resulting from this analysis included an inverse variance-covariance matrix of estimates computed at the maximum likelihood estimate and trait residuals based on covariate effects.

For the second step of the association analysis, the formetascore() function with option mmscore was chosen. This function applies a linear mixed effect regression model based on a score test for association, similar to the test described by Chen and Abecasis [26]. The score test incorporates trait residuals and allelic information for each SNP (coded as 0-2 according to the number of effect alleles per SNP and individual) as well as the inverse variance-covariance matrix of estimates. The score was computed for each SNP and follows an approximate chi-square distribution with 1 degree of freedom. Genetic association analysis was performed for all autosomes. Markers with a minor allele frequency (MAF) <0.05, a call rate <0.95 and/or a significant deviation from Hardy Weinberg Equilibrium $p(HWE)<10^{-6}$ were excluded after imputation. Manhattan and quantile-quantile plots were generated in R [27] using the manhattan() and QQ() functions by Stephen Turner [28].

Gene-based association

To determine association with hearing phenotypes on a gene rather than marker based level, the versatile gene-based test for GWAS (VEGAS)[29] was used. This freely available software (http://gump.qimr.edu.au/VEGAS/) combines the association results between a trait and all available markers within a gene (including 50kb of 3' and 5' untranslated regions) to calculate a combined p-value per gene. SNPs were mapped to 17787 genes according to the UCSC genome Browser human genome 18. After adjustment for multiple testing, a Bonferroni corrected significance threshold of $p<2.81x10^{-6}$ (0.05/17787) was considered genome-wide significant.

Functional Annotation of GWA results

All markers from the GWA analysis with a p-value below $5x10^{-6}$ were taken forward for functional annotation. Position within or near a gene and function of each selected marker was followed up using the SNP and copy number annotation tool (SCAN) [30] and UCSC Genome Browser (hg18) [31,32]. Regional Manhattan plots of the most highly associated SNPs ± 400 kb were created using Locus Zoom [33] for all loci harbouring suggestive significantly associated markers. Linkage disequilibrium information used in the Locus Zoom plots was based on the HapMap Phase 2, CEU sample. Genes harbouring associated markers were followed up in the literature.

All genes from the gene-based association analysis for SRT, PTA and PCs with a p-value $<10^{-3}$ were taken forward for an enrichment analysis in GeneMania [34]. GeneMania tests for interaction between genes and proteins and allows for enrichment analysis of a given gene list in various gene ontology (GO) terms. Enrichment is calculated in a hypergeometric test for enrichment. Significance of enrichment is expressed as a false discovery rate (FDR) Q-value estimated based on the Bejamini-Hochberg procedure [35]. Genes that had been associated with several hearing phenotypes were only included once in the analysis, giving a gene list of 52 unique genes associated with at least one of four hearing phenotypes (age and gender-adjusted SRT residuals, age-adjusted PTA, PC1 and PC2 residuals). Up to 20 related genes were allowed to be added by GeneMania to the list of 52 query genes based on previous functional annotations. The list of query (and related) genes with a specific GO annotation was compared to the number of all genes in the genome with the same annotation term. Functional enrichment analysis was limited to gene ontology annotation terms based on molecular function.

GWAS validation study

Validation of association with SRTs was sought in a separate sample with PTA data. Individuals for the SRT GWAS validation were selected if they had genotyping and PTA data available, but not been included in the SRT discovery GWAS. Age-adjusted PTA residuals were selected as validation phenotype, as they showed the strongest correlation with SRTs in chapter 4.

Likewise, validation of association with age-adjusted PC and PTA residuals was sought in a sample of volunteers with speech-in-noise and genotyping data, who had not been part of the pure-tone audiogram discovery GWASs previously.

Validation was sought on a marker (including all SNPs with $p<5x10^{-5}$ in the respective discovery GWAS) and gene level (including all associated genes from the gene-based

analysis with $p<10^{-3}$). Validation of association was defined as a nominally significant association ($p<0.05$) with the same marker or gene following the same direction of effect in case of the marker-based validation.

Results

Study participants

For the GWA analysis, all volunteers with hearing data (pure-tone audiometry or speech-in-noise test) and available genotyping data were selected. 1028 individuals with pure-tone audiometry and 1214 with speech-in-noise data fulfilled these criteria. Participants for the PCs and PTA GWASs were all female, whereas the group selected for the SRT GWAS was primarily female, but also included a minority of male subjects (108 males and 1106 females). Age of participants at pure-tone audiogram and speech-in-noise test ranged between 41-86 and 20-83 years, respectively. In accordance with the wider age range, mean age was slightly lower for the SRT GWAS sample compared to the PC1 and PC2 GWAS samples (56.85±11.29 SD compared to 61.98±8.29 SD). The characteristics of the two different study groups used in the discovery GWASs are summarized in Table 28.

Table 28 Characteristics of individuals with genotyping and hearing data

measure of hearing ability	n	gender (M/F)	age [years] range	age [years] mean ± SD	phenotype mean ± SD
PTA	1028	0/1028	41-86	61.98 ±8.29	22.4795 ±9.8740
PTA age-adjusted					-0.0584 ±8.1381
PC1 age-adjusted					-0.0747 ±1.8770
PC2 age-adjusted					0.0521 ± 1.3587
SRT age and gender adjusted	1214	108/1106	20-83	56.85 ±11.29	3.22x10-5 ±0.0033

Participants for the GWASs of ARHI and hearing function were recruited from two different datasets from TwinsUK, the pure-tone audiometry and speech-in-noise test data collection. Pure-tone audiometry was summarised as pure-tone averages (PTA) and principal components (PC1; PC2). Both cohorts are characterised by their sample size (n), the gender distribution (M=male; F=female), age of participants at hearing test, presented as age range and mean age ± standard deviation from the mean (SD). For each phenotype, the original values (PTA unadjusted), age-adjusted residuals (age-adjusted PTA, PC1 and PC2) or age-and gender adjusted residuals (SRT) are presented as mean ± standard deviation (SD).

Marker-based GWAS

After exclusion of markers with too low minor allele frequency (MAF<0.05), deviation from HWE (p(HWE)<10^{-4}) and reduced call rate (call rate<0.95), 1328646 SNPs (genotyped and imputed) remained for the hearing ability with age GWAS and were tested for association with age-adjusted PTA, PC1 and PC2 residuals as well as original PTA values. For each GWA study, the strongest association signals at a significance level below $5x10^{-6}$ ($p<5x10^{-6}$) were presented, in accordance with the universally excepted significance threshold of $p<5x10^{-6}$ for suggestive genome-wide significant associations [12].

Age-adjusted PTA

Age-adjusted PTA residuals were significantly associated ($p<5x10^{-6}$) with 2 markers on chromosome (chr) 14. The most significant association with age-adjusted PTA residuals was observed for SNP rs4898904 on chr 14 (beta±se=-0.3315±0.0709, p=2.90E-06). The two significantly associated markers on chr 14 were imputed. All SNPs associated with age-adjusted PTA residuals at a significance threshold of $p<5x10^{-6}$ are listed in order of their significance of association in Table 29. None of the associated markers reached genome-wide significance levels ($p<5x10^{-8}$).

The Manhattan plot (Figure 21) showed the significance of association with age-adjusted PTA residuals for each marker according to its position in the genome. For the associated markers on chromosome 14, 3 highly associated SNPs at the same locus could be detected. Due to LD between nearby markers, this association peak was expected. The QQ plot (Figure 22) compared the distribution of observed p-values against significance levels expected under the null hypothesis of no association (expected p-value). It could be seen that for lower extremes of the p-value distribution ($p<10^{-5}$), less significant associations than expected under the null hypothesis were detected.

Table 29 GWAS Results for age-adjusted PTA

SNP	chr	position	allele1 allele2	MAF	n	beta ± SE	p	gene feature	left gene right gene
rs4898904	14	56086782	C T	0.13	985	-0.33 ± 0.07	2.90E-06	NA NA	*PELI2* *C14orf101*
rs4898903	14	56086588	A G	0.13	991	-0.33 ± 0.07	3.57E-06	NA NA	*PELI2* *C14orf101*

Two single nucleotide polymorphisms (SNPs) were significantly associated ($p<5x10^{-6}$) with age-adjusted PTA residuals. The age-adjusted PTA GWAS results are described by their rs-number (SNP), the chromosome (chr) and base-pair location (position), non-effect allele (allele1) and effect allele (allele2), minor allele frequency (MAF), the number of individuals with genotyping or imputation data for the respective SNP (n), the effect size (beta) and corresponding standard error (SE) and significance of association (p). Mapping information for each SNP is specified by genes at the respective locus (gene) and surrounding the locus (left and right gene) as well as feature of the SNP position within a gene (feature). All SNPs were mapped to the forward (+) strand.

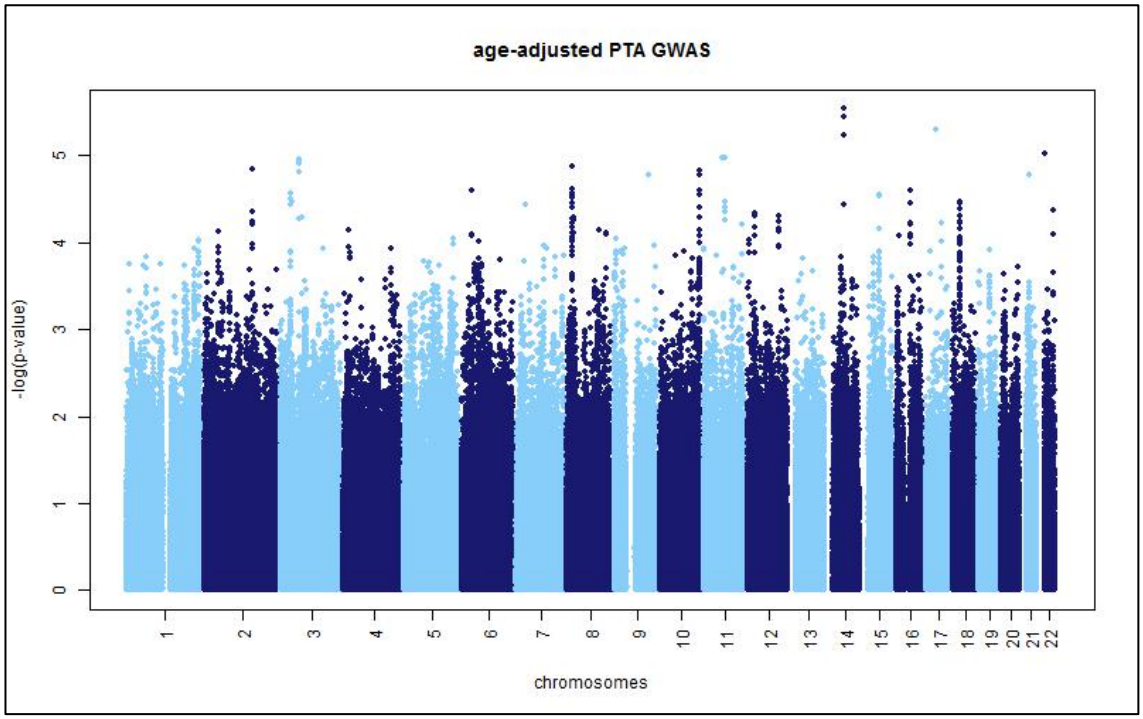


Figure 21 Manhattan plot for age-adjusted PTA GWAS

The Manhattan plot depicts the chromosomal location of genetic markers versus their significance of association with age-adjusted PTA residuals. Significance of association is measured as the negative logarithm of the p-value. Significant associations ($p<5x10^{-6}$) were found on chr 14.

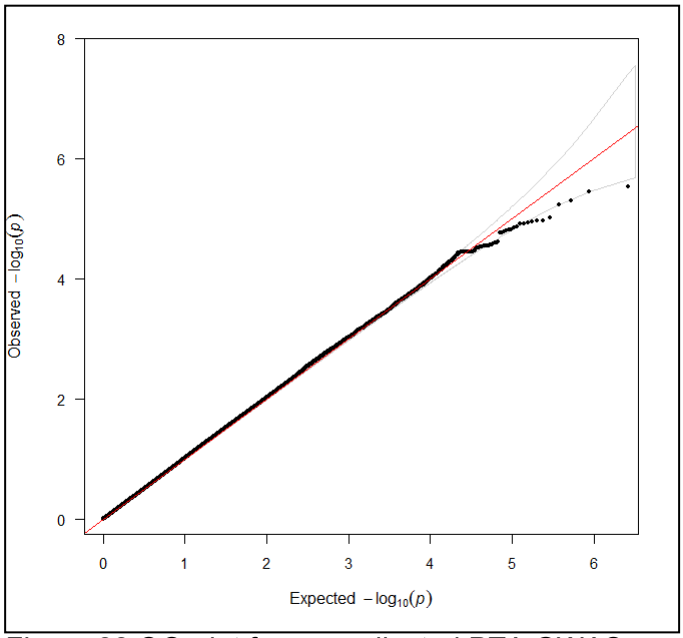


Figure 22 QQ-plot for age-adjusted PTA GWAS

The quantile-quantile plot depicts the expected significance of association under the null hypothesis of no association versus the observed significance (p) for the age-adjusted PTA GWAS. Significance of association is measured as the negative logarithm of the p-value.

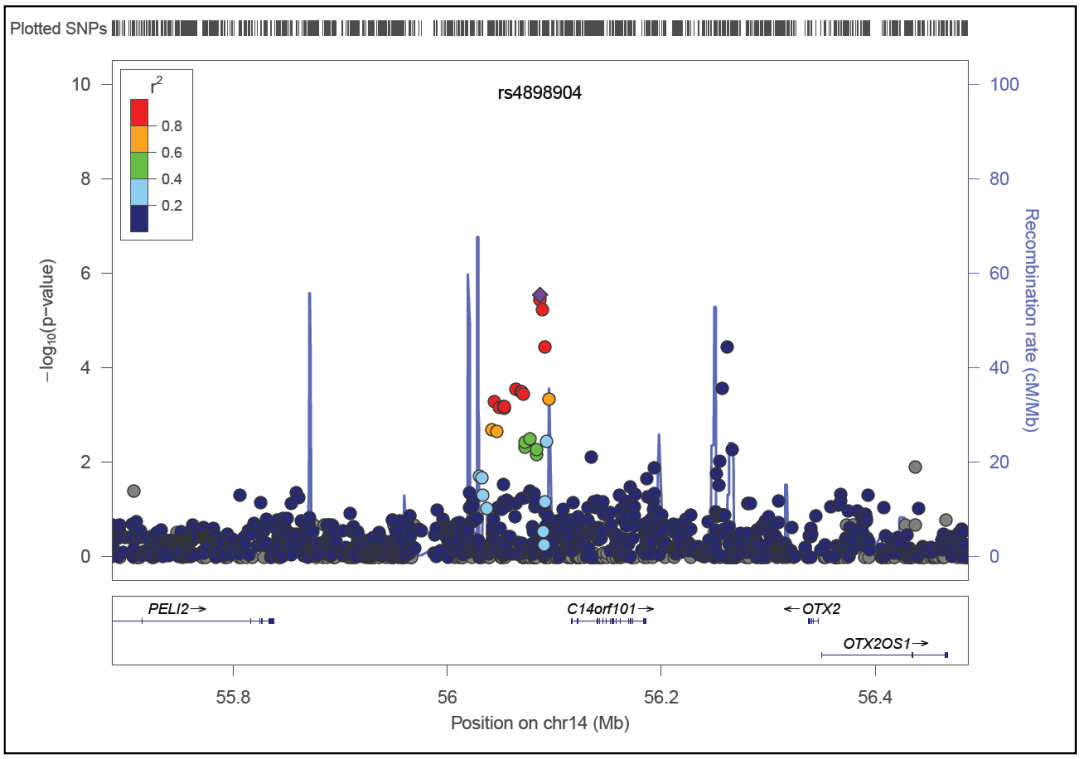


Figure 23 Locus zooms for age-adjusted PTA GWAS results on chr 14

The locus zoom depicts the location of genetic markers versus their significance of association with age-adjusted PTA values. Significance of association is measured as the negative logarithm of the p-value. Genes located in the area 400kb up-and downstream of the reference SNP (violet diamond) are displayed below the x-axis. The colour of each genetic marker indicates its correlation with the reference SNP. A legend for the correlation colour scheme is shown in the upper left corner. Recombination rate is highlighted as light blue peaks.

Associated markers on chr 14 mapped to a not previously annotated region near *C14orf101.* A recombination event (blue peaks in the Locus zoom) between the associated markers and the coding region of open reading frame *C14orf101* explains the lack of LD between the reference SNP and neighbouring loci.

PTA GWAS

In chapter 3, an increase in heritability was observed for participants of higher age groups. It was thus hypothesized that age plays an important role in ARHI and that adjustment for age in GWAS might reduce the effect size of genetic variants under age-related gene expression regulation. To determine the effect of age-adjustment on ARHI GWAs, GWASs for both age-adjusted PTA residuals and unadjusted PTAs were performed.

Unadjusted PTA values were significantly associated ($p<5x10^{-6}$) with 4 markers located on chr 6 and 10. These most highly associated markers are listed in Table 30 in order of significance of association. All associated markers were located in intronic regions of genes. The corresponding Manhattan plot (Figure 24) shows 3 defined association

peaks on each chr 6, 8 and 10. Like for the GWAS of age-adjusted PTA residuals, the QQ plot (Figure 25) showed a negative deviation of the observed p-values from the expected distribution of p-values for the high significance levels. In general, significance levels of association did not exceed the ones observed for the age-adjusted PTA GWAS ($p=3.52x10^{-6}$ compared to $2.90x10^{-6}$ after age-adjustment) and the most highly associated markers did not overlap.

Table 30 GWAS Results for PTA

SNP	chr	position	allele1 allele2	MAF	n	beta ± SE	p	gene feature	left gene right gene
rs7904722	10	131851881	C T	0.38	1028	-0.22 ±0.05	3.52E- 06	*GLRX3* intron	*LOC387723* *TCERG1L*
rs17297984	10	131857683	T A	0.37	1009	-0.22 ±0.05	4.04E- 06	*GLRX3* intron	*LOC387723* *TCERG1L*
rs10499151	6	129473312	C T	0.11	996	-0.35 ±0.08	4.13E- 06	*LAMA2* intron	*MESTP1* *LOC643778*
rs4001969	10	131842055	T C	0.38	1026	-0.22 ±0.05	4.91E- 06	*GLRX3* intron	*LOC387723* *TCERG1L*

Four single nucleotide polymorphisms (SNPs) were significantly associated ($p<5x10^{-6}$) with PTA values. The PTA GWAS results are described by their rs-number (SNP), the chromosome (chr) and base-pair location (position), non-effect allele (allele1) and effect allele (allele2), minor allele frequency (MAF), the number of individuals with genotyping or imputation data for the respective SNP (n), the effect size (beta) and corresponding standard error (SE) and significance of association (p). Mapping information for each SNP is specified by genes at the respective locus (gene) and surrounding the locus (left and right gene) as well as feature of the SNP position within a gene (feature). All SNPs were mapped to the forward (+) strand.

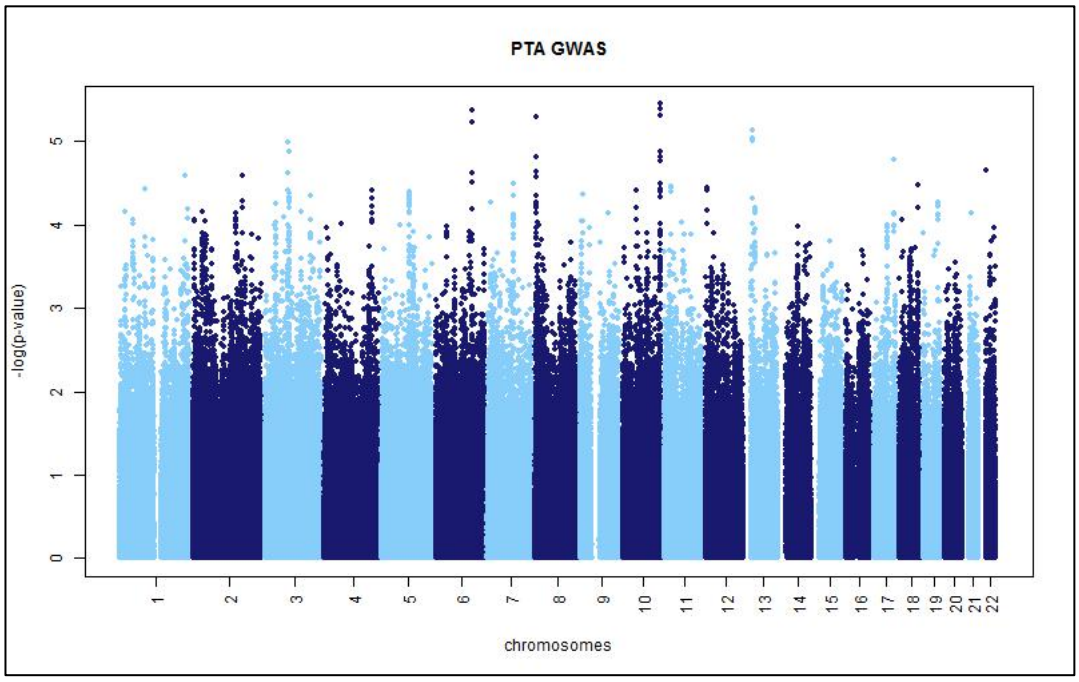


Figure 24 Manhattan plot for PTA GWAS

The Manhattan plot depicts the chromosomal location of genetic markers versus their significance of association with unadjusted PTAs. Significance of association is measured as the negative logarithm of the p-value. Significant associations ($p<5x10^{-6}$) were found on chr 6 and 10.

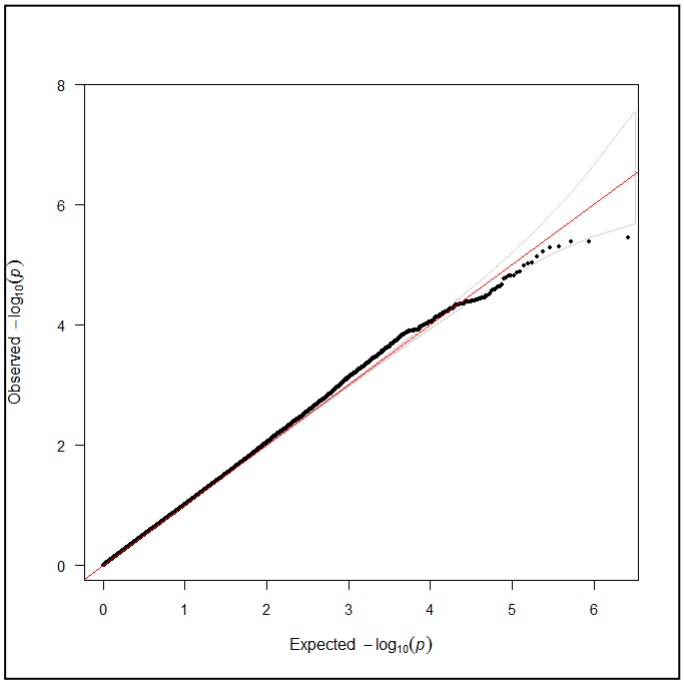


Figure 25 QQ-plot for PTA GWAS

The quantile-quantile plot depicts the expected significance of association versus the observed significance (p) for the unadjusted PTA GWAS. Significance of association is measured as the negative logarithm of the p-value.

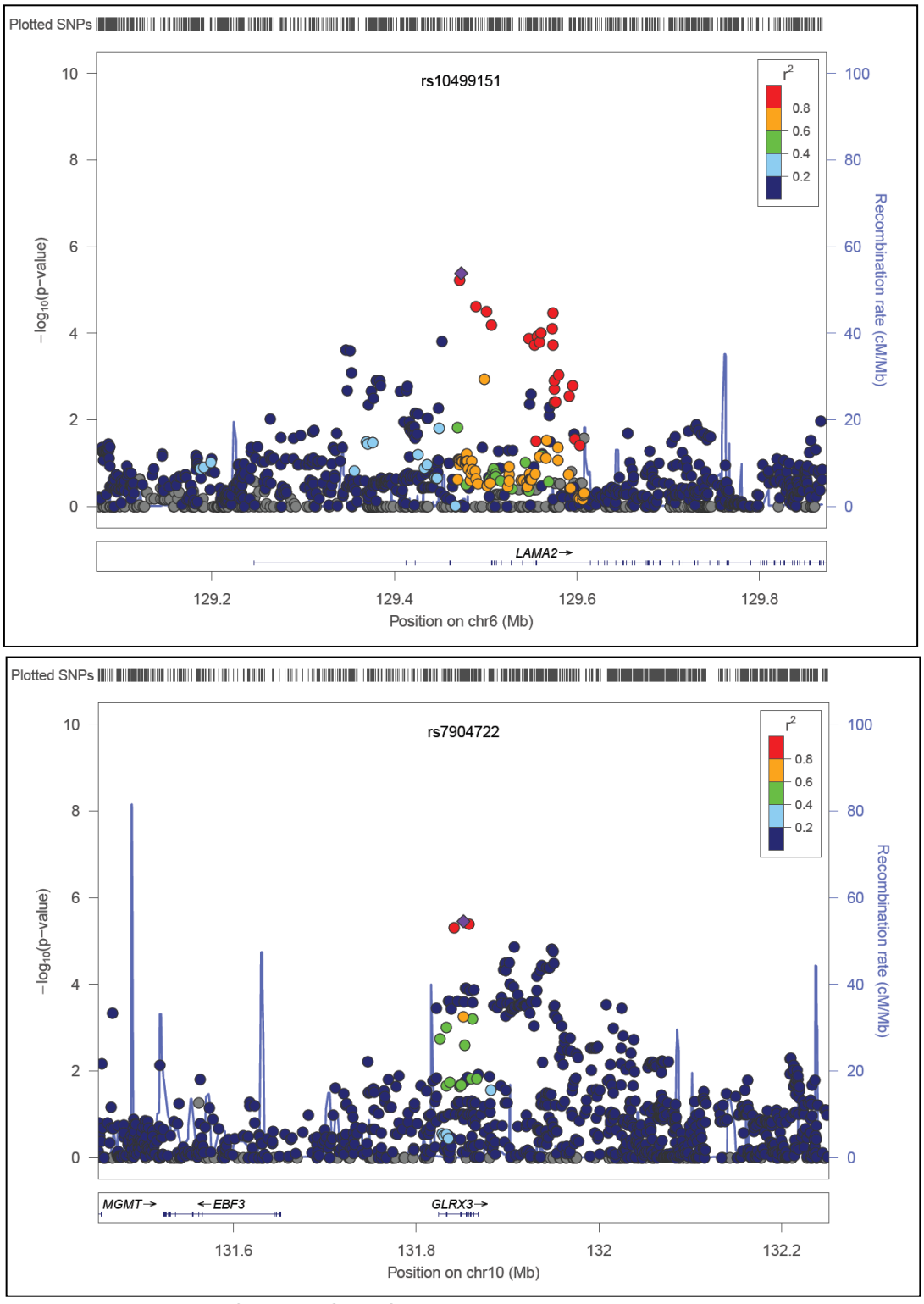


Figure 26 Locus zoom for PTA GWAS results on chr 6 and 10

The locus zoom depicts the location of genetic markers versus their significance of association with PTA values. Significance of association is measured as the negative logarithm of the p-value. Genes located in the area 400kb up-and downstream of the reference SNP (violet diamond) are displayed below the x-axis. The colour of each genetic marker indicates its correlation with the reference SNP. A legend for the correlation colour scheme is shown in the upper left corner. Recombination rate is highlighted as light blue peaks.

Associated markers on chr 6 were located in an intron of the Laminin Alpha 2 (LAMA2) gene. The locus zoom of chr 6 (Figure 26) shows that the reference SNP rs10499151 is in high LD ($R^2 \geq 0.8$) with various other associated SNPs at this locus, with LD extending over an area of 0.1 Mb.

PTA values were further associated with markers mapping to introns of the Glutaredoxin 3 (*GLRX3*) gene on chromosome 10 (Figure 26). GLRX3 encodes a member of the glutaredoxin family, which have the ability to reduce different substrates upon use of glutathione as a cofactor.

PC1 GWAS

After quality control, 4 of 1329738 total markers showed significant association with PC1 at a significance level of $p<5x10^{-6}$. Of these, one marker had been genotyped (rs5748636), while the remaining three had been imputed according to HapMap Phase 2 data. The strongest association signal was observed for rs5748636 (beta± se= 0.266356±0.053771, p=7.29E-07). Significantly associated SNPs mapped to introns or intergenic regions on chr 10 and 22 and are listed in Table 31 in order of association strength. The Manhattan plot of PC1 GWAS results (Figure 27) shows a defined association peak on chr 10. The association on chr 22 appears to result from association with a single marker (rs5748636). According to the QQ-plot, the distribution of observed associations closely follows the expected distribution of p-values and only deviates slightly from the expected distribution for low p-values.

Table 31 GWAS Results for age-adjusted PC1

SNP	chr	position	allele1 allele2	MAF	n	beta ± SE	p	gene feature	left gene right gene
rs5748636	22	15655394	G A	0.26	1028	0.27 ± 0.05	7.29E-07	*XKR3* intron	*VWFP* *CECR8*
rs7069495	10	131950097	G C	0.10	1010	-0.37 ± 0.08	2.20E-06	NA NA	*GLRX3* *TCERG1L*
rs1999109	10	131948198	A G	0.10	1012	-0.37 ± 0.08	2.35E-06	NA NA	*GLRX3* *TCERG1L*
rs6482783	10	131950554	G A	0.10	1000	-0.37 ± 0.08	3.15E-06	NA NA	*GLRX3* *TCERG1L*

Four single nucleotide polymorphisms (SNPs) were significantly associated ($p<5x10^{-6}$) with age-adjusted PC1 residuals. The age-adjusted PC1 GWAS results are described by their rs-number (SNP), the chromosome (chr) and base-pair location (position), non-effect allele (allele1) and effect allele (allele2), minor allele frequency (MAF), the number of individuals with genotyping or imputation data for the respective SNP (n), the effect size (beta) and corresponding standard error (SE) and significance of association (p). Mapping information for each SNP is specified by genes at the respective locus (gene) and surrounding the locus (left and right gene) as well as feature of the SNP position within a gene (feature). All SNPs were mapped to the forward (+) strand.

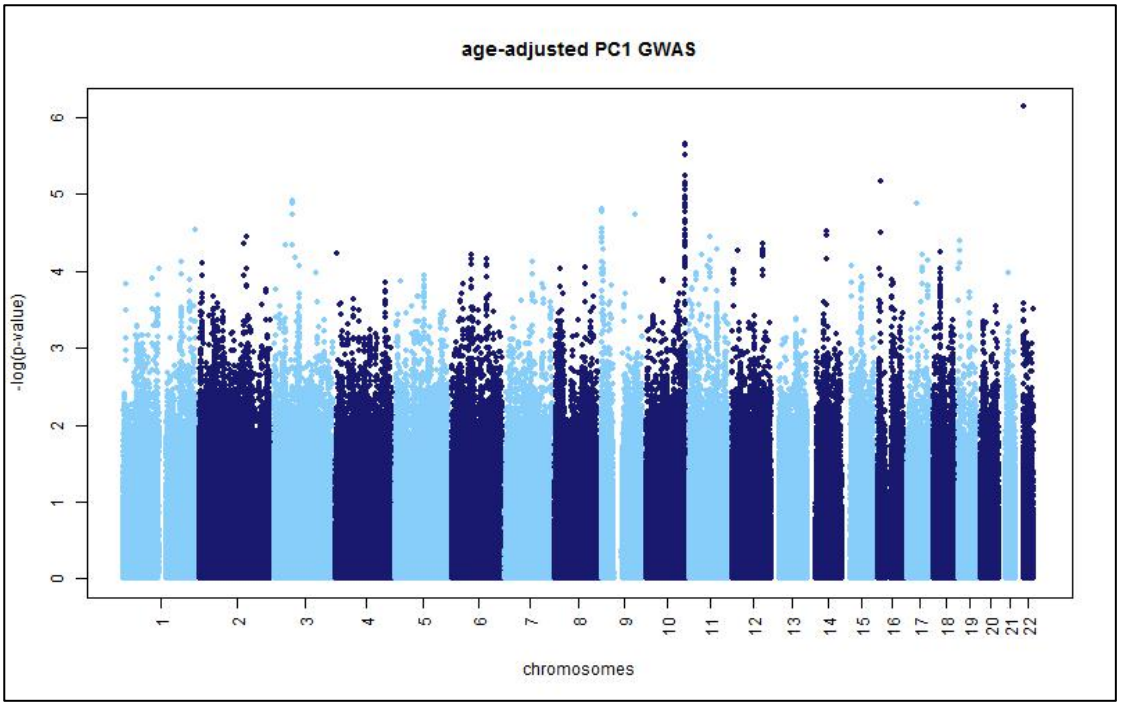


Figure 27 Manhattan plot for age-adjusted PC1 GWAS

The Manhattan plot depicts the chromosomal location of genetic markers versus their significance of association with age-adjusted PC1 residuals. Significance of association is measured as the negative logarithm of the p-value. Significant associations ($p<5x10^{-6}$) were found on chromosomes 10 and 22.

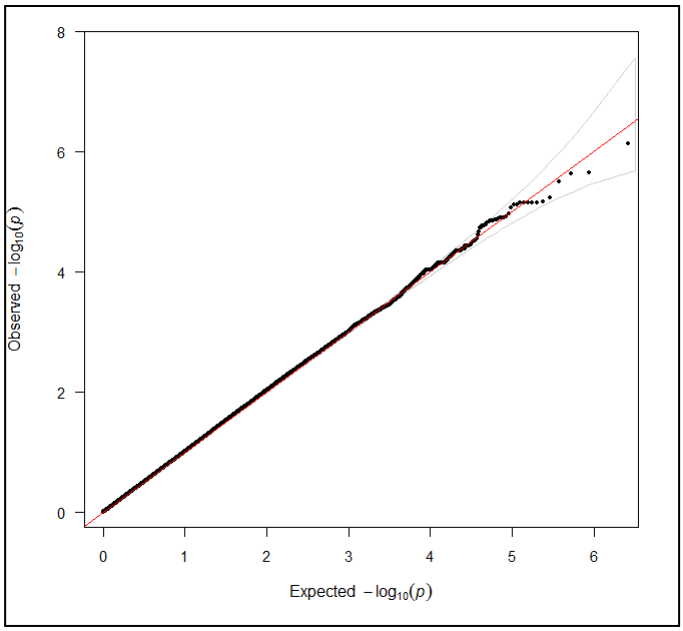
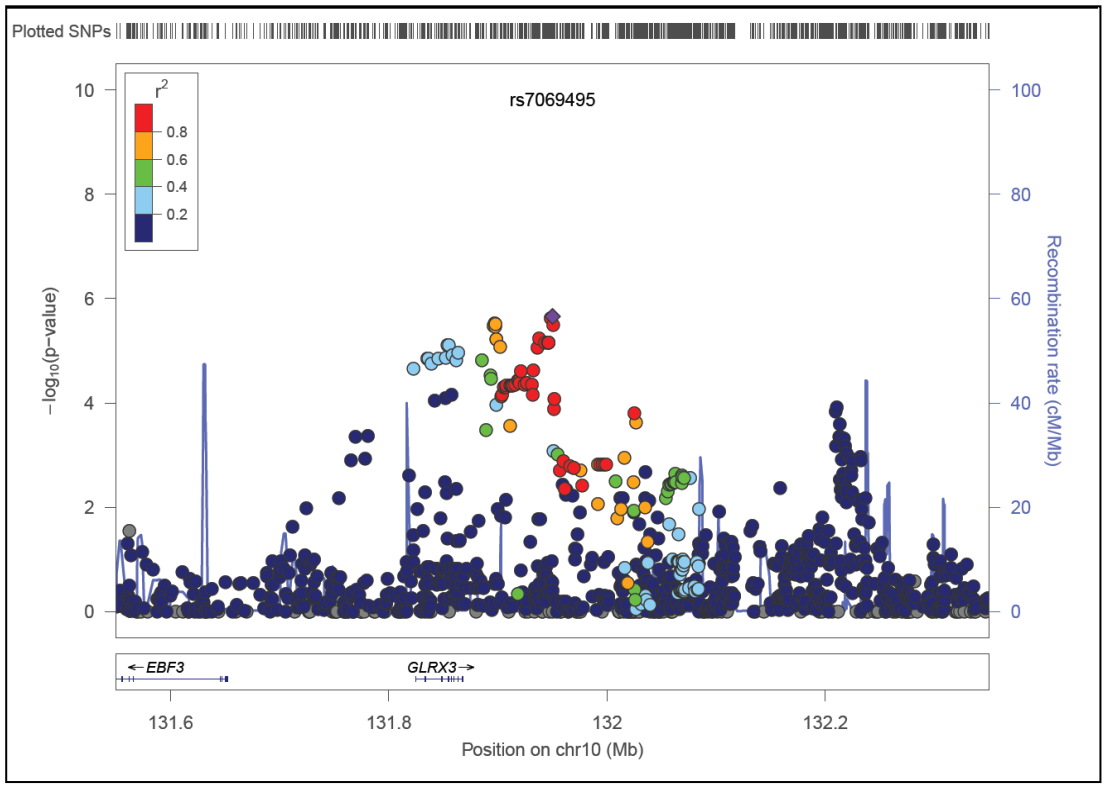


Figure 28 QQ-Plot for age-adjusted PC1 GWAS

The quantile-quantile plot depicts the expected significance of association versus the observed significance (p) for the age-adjusted PC1 GWAS. Significance of association is measured as the negative logarithm of the p-value.



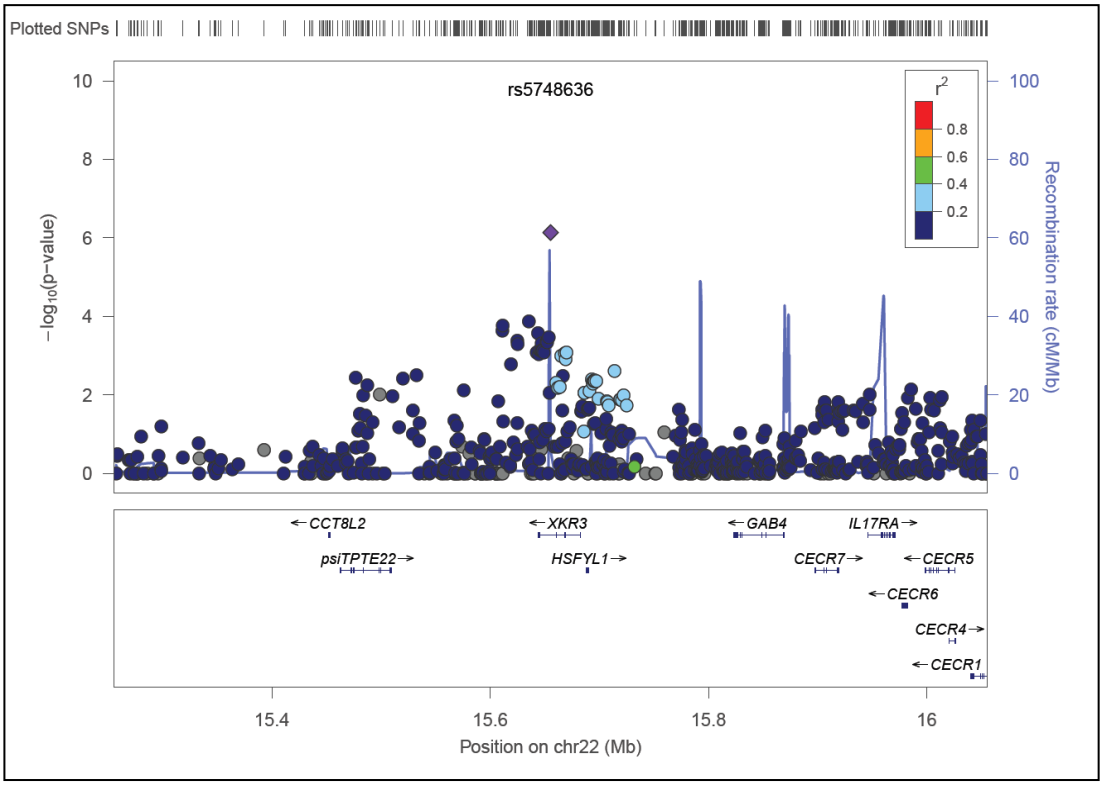


Figure 29 Locus zoom for age-adjusted PC1 GWAS results on chr 10 and 22

The locus zoom depicts the location of genetic markers versus their significance of association with PC1 values. Significance of association is measured as the negative logarithm of the p-value. Genes located in the area 400kb up-and downstream of rs7069495 are displayed below the x-axis. The colour of each genetic marker indicates its correlation with the reference SNP rs7069495. A legend for the correlation colour scheme is shown in the upper left corner. Recombination rate is highlighted as light blue peaks.

The most highly associated SNP was located in an intron of the XK, Kell Blood Group Complex Subunit-Related Family, Member 3 (*XKR3*) on chr 22 (Figure 29). As indicated by the Manhattan plot and confirmed in the locus zoom, the reference SNP rs5748636 maps closely to a recombination break point and is only in moderate LD with surrounding SNPs (Figure 29). Association for the same SNP was detected in the age-adjusted PTA GWAS.

Further associated markers were located in non-coding regions and down-stream of the Glutaredoxin 3 (*GLRX3*) gene. The most strongly associated markers on chr 10 map to introns of *GLRX3*, but are in strong LD with SNPs downstream of the *GLRX3* coding region (Figure 29). Similar association signals for *GLRX3* have been observed in the PTA GWAS.

PC2 GWAS

Age-adjusted PC2 residuals were significantly associated ($p<5x10^{-6}$) with 11 SNPs, which are listed according to their strength of association in Table 32. The strongest association of age-adjusted PC2 residuals was observed for rs4747375 (beta± se=0.3088± 0.0625, p=7.80E-07) on chr 10. Two significantly associated SNPs were genotyped and located at the same locus on chr 10. Most SNPs significantly associated with PC2 mapped to non-coding regions. The Manhattan plot of PC2 GWAS results (Figure 30) showed two defined association peaks on chr 7 and 10. The -plot (Figure 31) shows neither deflation nor inflation in observed p-values.

Table 32 GWAS results for age-adjusted PC2

SNP	chr	position	allele1 allele2	MAF	n	beta ± SE	p	gene feature	left gene right gene
rs4747375	10	19829342	T G	0.18	1010	0.31 0.06	7.80E-07	*LOC100128641* intron	*C10orf112* *PLXDC2*
rs2358412	10	19846259	T C	0.11	1013	0.35 0.07	2.05E-06	*LOC100128641* intron	*C10orf112* *PLXDC2*
rs10494867	10	19849680	C A	0.11	1027	0.35 0.07	2.06E-06	*LOC100128641* intron	*C10orf112* *PLXDC2*
rs7921915	10	19851760	G T	0.11	1027	0.35 0.07	2.06E-06	*LOC100128641* intron	*C10orf112* *PLXDC2*
rs7895790	10	19846022	G A	0.11	1012	0.35 0.07	2.08E-06	*LOC100128641* intron	*C10orf112* *PLXDC2*
rs3864833	10	19850180	T C	0.11	1024	0.35 0.07	2.74E-06	*LOC100128641* intron	*C10orf112* *PLXDC2*
rs7907741	10	19851717	A T	0.11	1024	0.35 0.07	2.74E-06	*LOC100128641* intron	*C10orf112* *PLXDC2*
rs7800962	7	3512416	G A	0.31	983	0.24 0.05	3.01E-06	*SDK1* intron	*LOC100129603* *LOC730351*
rs3852468	10	19840498	T C	0.11	1004	0.35 0.08	3.28E-06	*LOC100128641* intron	*C10orf112* *PLXDC2*
rs6462121	7	3513830	G C	0.31	996	0.24 0.05	4.27E-06	*SDK1* intron	*LOC100129603* *LOC730351*
rs1412779	10	19838319	T G	0.11	1003	0.34 0.08	4.78E-06	*LOC100128641* intron	*C10orf112* *PLXDC2*

Eleven single nucleotide polymorphisms (SNPs) were significantly associated ($p<5x10^{-6}$) with age-adjusted PC2 residuals. The age-adjusted PC2 GWAS results are described by their rs-number (SNP), the chromosome (chr) and base-pair location (position), non-effect allele (allele1) and effect allele (allele2), minor allele frequency (MAF), the number of individuals with genotyping or imputation data for the respective SNP (n), the effect size (beta) and corresponding standard error (SE) and significance of association (p). Mapping information for each SNP is specified by genes at the respective locus (gene) and surrounding the locus (left and right gene) as well as feature of the SNP position within a gene (feature). All SNPs were mapped to the forward (+) strand.

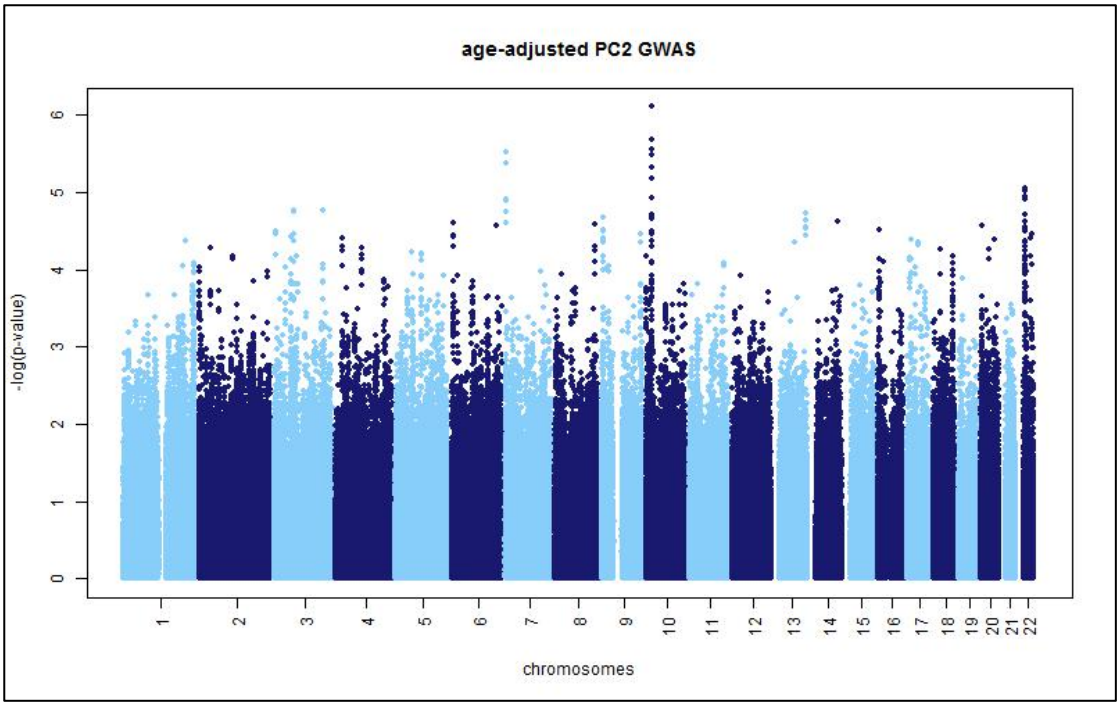
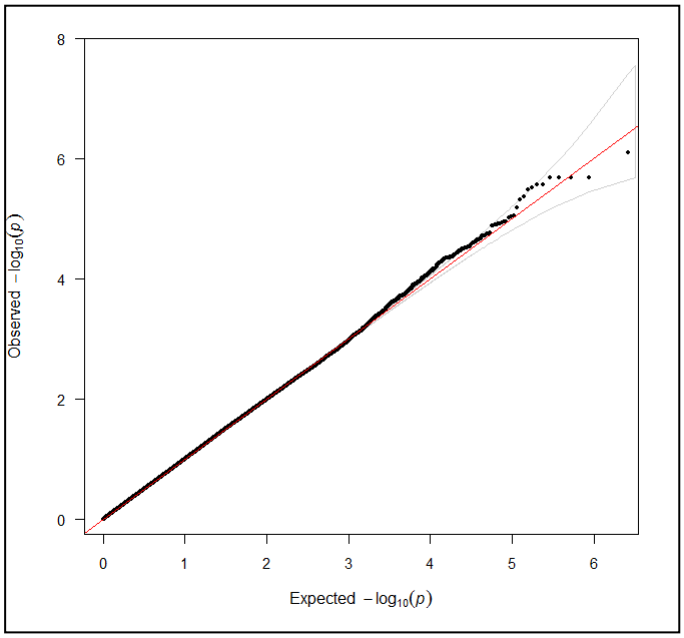


Figure 30 Manhattan plot for age-adjusted PC2 GWAS

The Manhattan plot depicts the chromosomal location of genetic markers versus their significance of association with age-adjusted PC2 residuals. Significance of association is measured as the negative logarithm of the p-value. Significant associations ($p<5x10^{-6}$) were found on chromosomes 7 and 10.

Figure 31 QQ-Plot for age-adjusted PC2



The quantile-quantile plot depicts the expected significance of association versus the observed significance (p) for the age-adjusted PC2 GWAS. Significance of association is measured as the negative logarithm of the p-value.

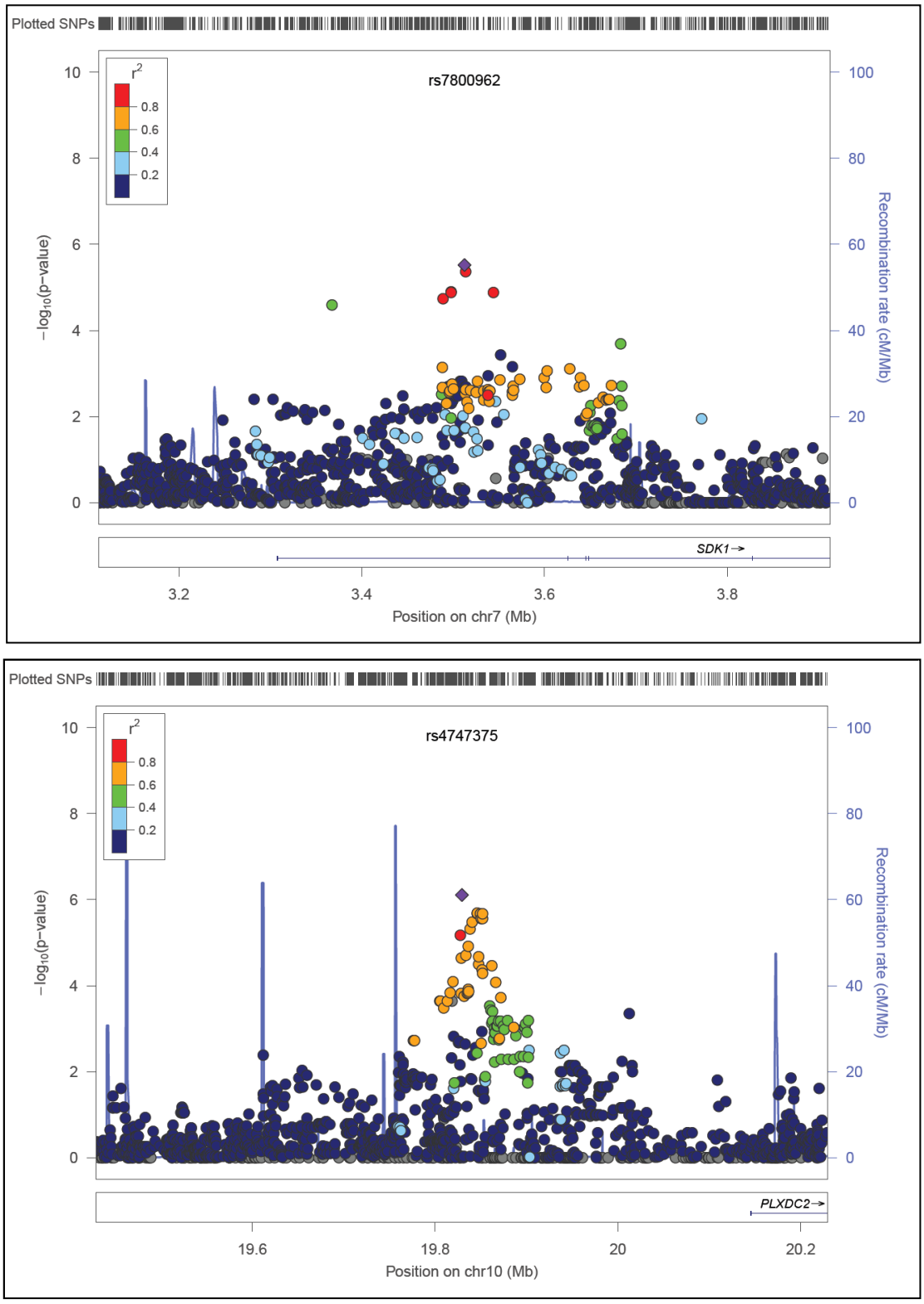


Figure 32 Locus zoom for PC2 GWAS results on chr 7 and 10

The locus zoom depicts the location of genetic markers versus their significance of association with PC2 values. Significance of association is measured as the negative logarithm of the p-value. Genes located in the area 400kb up-and downstream of the reference SNP (violet diamond) are displayed below the x-axis. The colour of each genetic marker indicates its correlation with the reference SNP. A legend for the correlation colour scheme is shown in the upper left corner. Recombination rate is highlighted as light blue peaks.

Significant associations on chr 7 (Figure 32) mapped to introns of Sidekick Cell Adhesion Molecule 1 (*SDK1*) gene. The reference SNP rs7800962 was in strong LD with surrounding SNPs located in introns of *SDK1*.

Associated markers on chr 10 (Figure 32) mapped to the LOC100128641 locus upstream of the Plexin Domain Containing 2 (*PLXDC2*) gene. The reference SNP rs4747375 was in moderate LD with surrounding genetic markers.

SRT GWAS

After quality control, 1344384 SNPs were tested for association with age and gender adjusted SRT residuals. Four genetic markers showed significant ($p<5x10^{-6}$) associations. These markers mapped to four different chromosomes and three of them were genotyped. The Manhattan plot (Figure 33) shows one defined association peak on chr 13 and associations with single markers on chr 1, 2 and 14. The QQ-plot (Figure 34) showed a slight deflation for highly significant associations.

Table 33 GWAS results for age-and gender adjusted SRT

SNP	chr	position	allele1 allele2	MAF	n	beta ± SE	p	gene feature	left gene right gene
rs28433318	2	21791	G A	0.07	1193	-0.37 0.08	2.55E-06	NA NA	NA *FAM110C*
rs9557794	13	101498211	T G	0.35	1170	0.20 0.04	3.67E-06	*FGF14* intron	*LOC100126007* *LOC100130168*
rs2290501	1	22047013	T G	0.33	1214	-0.20 0.04	3.74E-06	*HSPG2* intron	*LDLRAD2* *LOC440575*
rs13379210	14	66990924	T C	0.22	1192	0.23 0.05	4.84E-06	NA NA	*PLEK2* *C14orf83*

Four single nucleotide polymorphisms (SNPs) were significantly associated ($p<5x10^{-6}$) with age and gender-adjusted SRT residuals. The SRT GWAS results are described by their rs-number (SNP), the chromosome (chr) and base-pair location (position), non-effect allele (allele1) and effect allele (allele2), minor allele frequency (MAF), the number of individuals with genotyping or imputation data for the respective SNP (n), the effect size (beta) and corresponding standard error (SE) and significance of association (p). Mapping information for each SNP is specified by genes at the respective locus (gene) and surrounding the locus (left and right gene) as well as feature of the SNP position within a gene (feature). All SNPs were mapped to the forward (+) strand.

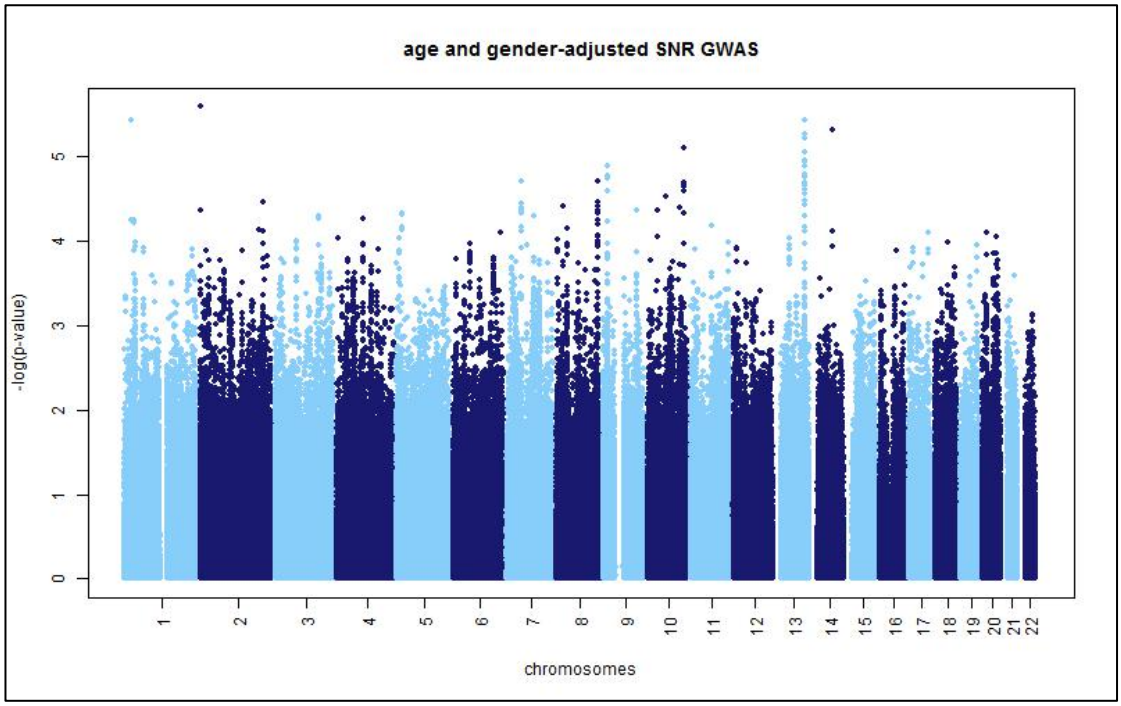


Figure 33 Manhattan plot for age and gender-adjusted SRT GWAS

The Manhattan plot depicts the chromosomal location of genetic markers versus their significance of association with age and gender-adjusted SRT residuals. Significance of association was measured as the negative logarithm of the p-value. Significant associations ($p<5x10^{-6}$) were found on chromosomes 1, 2, 13 and 14.

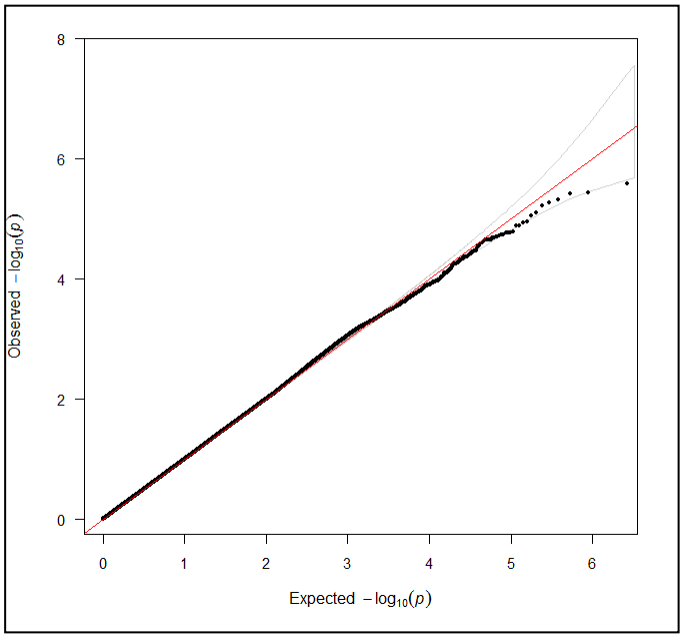


Figure 34 QQ-Plot for age- and gender adjusted SRT GWAS

The quantile-quantile plot depicts the expected significance of association versus the observed significance (p) for the age and gender-adjusted SRT GWAS. Significance of association is measured as the negative logarithm of the p-value.

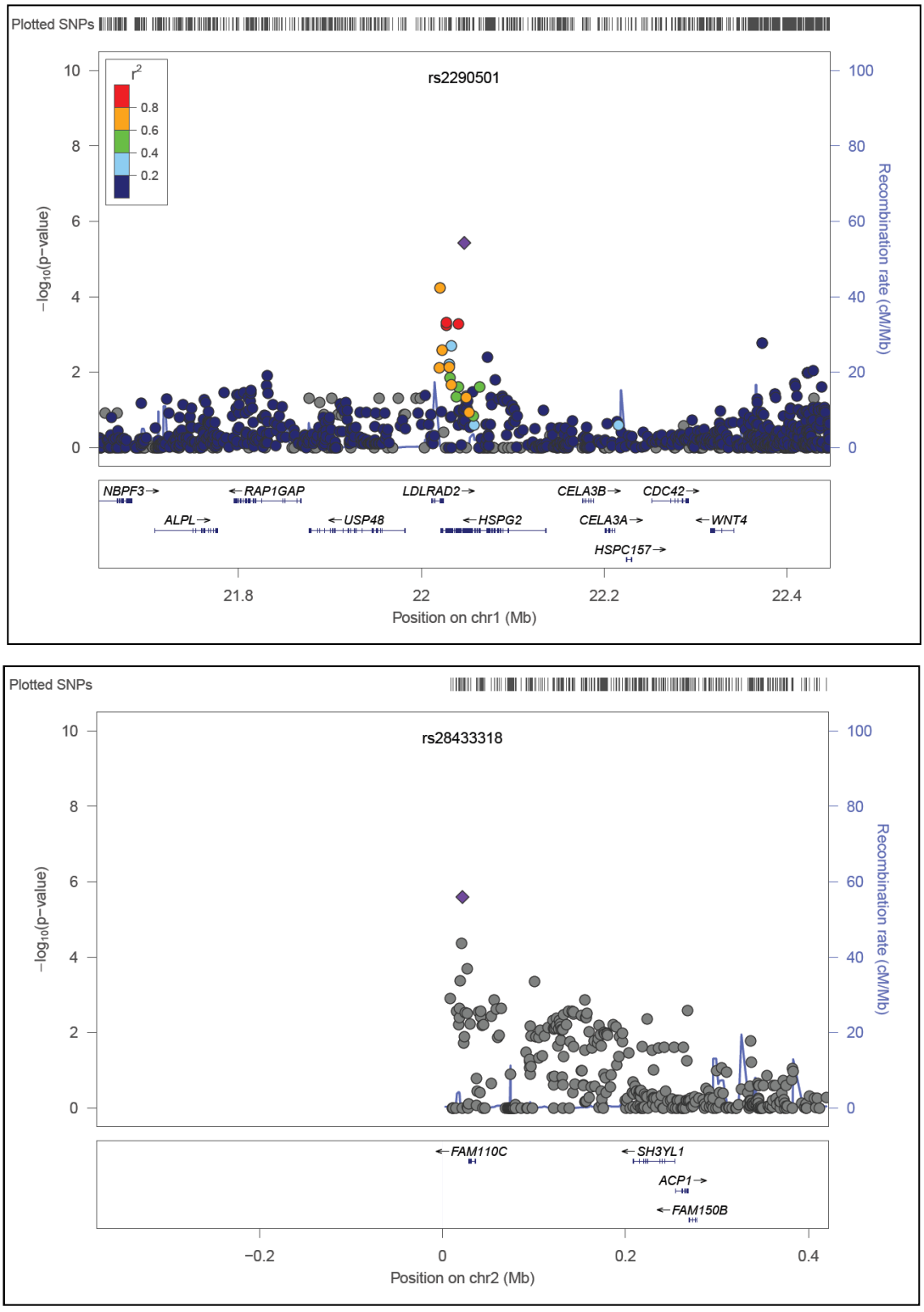


Figure 35 Locus zoom for age-and gender adjusted SRT GWAS results on chr 1 and 2

The locus zoom depicts the location of genetic markers versus their significance of association with SRT values. Significance of association is measured as the negative logarithm of the p-value. Genes located in the area 400kb up-and downstream of the reference SNP (violet diamond) are displayed below the x-axis. The colour of each genetic marker indicates its correlation with the reference SNP. A legend for the correlation colour scheme is shown in the upper left corner. There was no LD information available for the reference SNP on chr 2. Recombination rate is highlighted as light blue peaks.

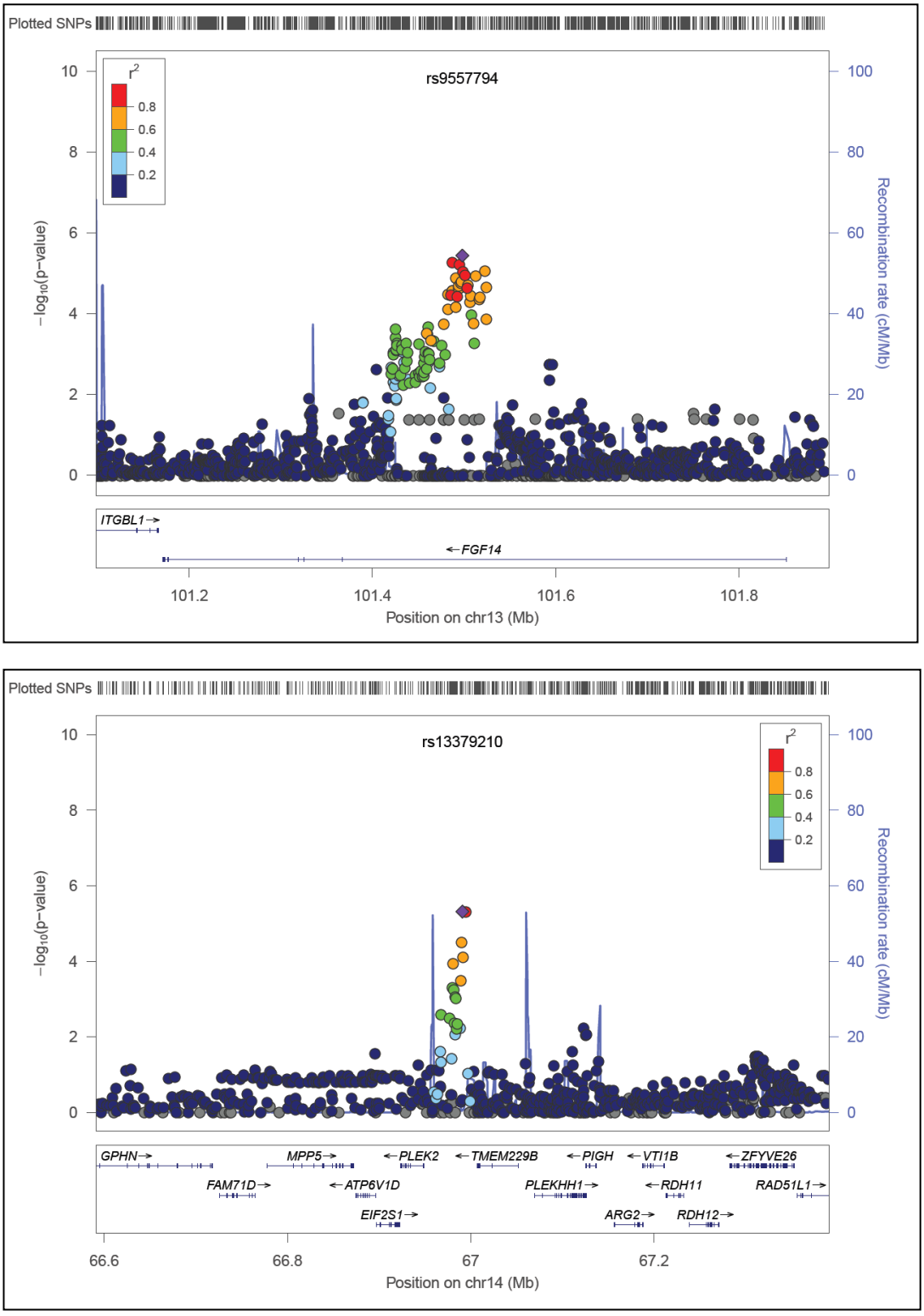


Figure 36 Locus zoom for age- and gender adjusted SRT GWAS on chr 13 and 14

The locus zoom depicts the location of genetic markers versus their significance of association with SRT values. Significance of association is measured as the negative logarithm of the p-value. Genes located in the area 400kb up-and downstream of the reference SNP (violet diamond) are displayed below the x-axis. The colour of each genetic marker indicates its correlation with the reference SNP. A legend for the correlation colour scheme is shown in the upper left corner. Recombination rate is highlighted as light blue peaks.

SNP rs2290501 associated with SRT residuals on chr 1 mapped to an intron of the HSPG2 gene (Figure 35). This SNP was in strong LD with 3 further SNPs included in the association study located in close proximity.

The most highly associated SNP ($p=2.5x10^{-6}$) was located at the beginning of the pter arm of chromosome 2 (Figure 35). No gene has been mapped to the exact location of this SNP and LD information for the reference SNP (rs28433318) was not available in the HapMap Phase 2 CEU dataset. However, rs28433318 was located in proximity of the Family with sequence similarity 110, member C gene (*FAM110C*).

In addition, alleles associated with SRT values were located in the Fibroblast growth factor 14 gene on chromosome 13 (Figure 36). The reference SNP rs9557794 and markers in strong LD with this SNP were all located in intron 1 of the FGF14 gene.

Hearing ability as measured by SRT was also associated with markers on chromosome 14 (Figure 36), upstream of the Plekstrin 2 (*PLEK2*) gene.

Gene-based association of ARHI and hearing function

Genes associated with ARHI or hearing function in the gene-based analysis did not reach genome-wide significance levels (p<2.81x10-6), however, all associated genes with a suggestive genome-wide significant association ($p<10^{-3}$) are listed in appendix chapter 5, Table 43.

In total, 32 genes were associated with hearing ability as measured by pure-tone audiometry ($p<10^{-3}$), while 22 genes showed significant associations with hearing function according to speech-in-noise reception. Some genes were associated with different hearing phenotypes (i.e. *GLRX3* and *MYO1E*).

The most highly associated genes ($p<10^{-4}$) included *FAM110C*, *GLRX3* and Dehydrogenase/Reductase Member 7C (*DHRS7C*).

Functional gene enrichment analysis

All genes associated with age and gender adjusted SRT residuals and age-adjusted PC and PTA residuals in the gene-based analysis were used in a functional enrichment analysis in GeneMANIA [34]. The resulting enrichment categories and significance of enrichment are presented in Table 34.

Most significant enrichment was seen for ephrin receptor gene ontologies. Four to 6 genes in the submitted gene list from the gene-based association results mapped to

these gene ontologies, with FDR Q-values for enrichment ranging from $1.83x10^{-7}$ to $1.04x10^{-3}$. Further gene ontologies enriched in the gene-based association results included cell-cell interaction terms and gene silencing ontologies.

Table 34 Results of the functional annotation of genes associated with hearing ability in GeneMania

Gene ontology term	FDR	Genes in query list	Genes in genome
ephrin receptor binding	1.83E-07	6	15
ephrin receptor signaling pathway	2.40E-06	6	24
ephrin receptor activity	1.04E-03	4	14
cell-substrate adhesion	2.72E-02	6	122
cell-matrix adhesion	8.05E-02	5	91
transmembrane receptor protein tyrosine kinase activity	1.08E-01	4	50
gene silencing by miRNA	2.19E-01	3	23
transmembrane receptor protein kinase activity	2.23E-01	4	67
posttranscriptional gene silencing by RNA	2.23E-01	3	26
posttranscriptional gene silencing	2.23E-01	3	26

Results of the functional gene enrichment analysis in GeneMANIA are presented by the different annotation terms (Gene ontology term). For each annotation term the false discovery rate Q-value (FDR) of enrichment, the number of genes involved in this term (genes in query list) and the number of genes in the genome associated with the corresponding annotation term are listed.

Validation of GWAS associations

670 volunteers with age-adjusted PTA residuals and 856 participants with SRT residuals were selected for the validation study. Both validation samples are described in more detail in Table 35.

Table 35 Characteristics of the validation sample

phenotype	n	gender (M/F)	age [years]		phenotype
			range	mean ±SD	mean ±SD
age-adjusted PTA	670	0/670	41-86	62.78 ±8.47	0.3839 ±8.2188
age-and gender adjusted SRT	856	108/748	20-83	55.37 ±12.17	-0.0001 ±0.0032

The validation sample was created from all participants with pure-tone audiogram or SRT data, who had been genotyped and not been included in the discovery GWAS of hearing function (age and gender-adjusted SRT) or ARHI (age-adjusted PTA, PC1 or PC2). Selected samples with PTA data were used for validation of the hearing function GWAS, while the validation samples for the ARHI GWAS included exclusively volunteers with SRT data. Both replication groups are characterised by their sample size, gender distribution (M=male; F=female), age at the respective hearing test and the age or age and gender-adjusted trait residuals. Phenotype residuals are presented as mean ± standard deviation from the mean (SD).

On a gene-based level, validation of association was observed for three genes that were associated with hearing ability as measured by pure-tone audiometry (age-adjusted PC and PTA residuals): Pannexin 1 (*PANX1*), Lysosomal Trafficking Regulator (*LYST*) and Wingless-Type MMTV Integration Site Family, Member 16 (*WNT16*) (Table 43).

Discussion

ARHI is a complex trait with no previously identified significant genome-wide significant associations [4,9,15,17] and only few suggestive significant genetic associations reliably replicated [14] despite moderate heritability estimates [36,37,38,39]. Here, the first GWASs of hearing ability with age in a purely female northern European cohort were described using PCs and the PTA calculated from pure-tone audiograms and SRTs from a speech-in-noise reception test in a mainly female cohort of wider age range (20-83 years). Despite moderate heritability estimates for hearing ability with age in TwinsUK, genome-wide significance levels could not be reached in this analysis, highlighting the problem of "missing heritability" [5].

Despite the lack of genome-wide significant associations, many suggestive significant associations were determined, including associations with genetic markers in genes *LAMA2*, *GLRX3*, *SDK1*, *HSPG2*, *FGF14* and *XKR3*. Laminin 2, associated with PTA values, is an important component of the basilar membrane. Mutations of *LAMA2* in humans are the cause of merosin-deficient muscular dystrophy. A laminin deficient mouse model of merosin-deficient muscular dystrophy showed increased auditory thresholds consistent with destruction of various cochlear structures [40]. *SDK1*, associated with PC2 residuals, has first been discovered in chick retinal ganglion cells [41] and is thought to be important for synapse formation via cell adhesion between the pre- and postsynaptic cells [42]. *HSPG2*, a gene associated with SRT residuals, encodes a heparan sulfate proteoglycan expressed in various mesenchymal tissues, including cartilage and basements membranes. *HSPG2* null mouse mutants show poorly developed inner and middle ear structures, indicating the importance of this gene and its protein product for ear development [43].

Although many highly associated markers mapped to genes previously linked to hearing disorders, the reduced significance threshold ($p<5x10^{-6}$) accepted here raised the risk of false positive associations. To limit this risk, a gene-based association analysis, which summarises association signals for genetic markers clustering at the

same gene or locus was applied. Accordingly, association signals for genes harbouring several moderate to highly associated markers were supported, whereas association signals for loci with single strongly associated markers were diluted. True positive associations with underlying causal variants often result in several neighbouring SNPs showing association with a trait due to underlying LD at the respective locus. The choice of this method is further supported by the fact that individuals affected by ARHI might show associations with different causal variants within the same gene, but not necessarily with the same marker. This method thereby might filter out likely false positive associations.

The resulting list of significantly associated ($p<10^{-3}$) genes comprised 52 unique genes including *FAM110C*, *GLRX3* and *DHRS7C*. Associations with markers in *FAM110C* and *GLRX3* had been highlighted in the marker-based analyses (association with SRT residuals and age-adjusted PTA and PC1 residuals, respectively) whereas the association with *DHRS7C* resulted primarily from the gene-based analysis. The *FAM110C* protein product has recently been involved in cell migration and filopodia formation [44] whereas mutations in glutaredoxin domains, similar to the ones found in *GLRX3,* have been associated with non-syndromic HL in humans [45] and inner ear dysfunction in the pirouette mouse [46]. *DHRS7C* is a short chain dehydrogenase reductase, which has been linked to heart failure. Dhrsc7 was significantly down-regulated under conditions of heart failure [47].

To functionally annotate genes from the gene-based analysis and thereby better understand underlying pathways, a gene enrichment analysis was performed. In this analysis, genes clustered in gene ontology categories for ephrin receptors, cell-cell interactions and gene silencing. Ephrin receptors (EFNA1, EFNA3 and EFNA4) were associated with PC2 residuals in the gene-based analysis. Ephrins and ephrin-receptors were shown to play an essential role in afferent type 1 and type 2 spiral ganglion neuron synapse formation with inner and outer hair cells [48]. Cell-cell interaction in the cochlea is indispensable to create a tight seperation between the different chambers of the cochlea and maintain functionality of the sensory hair cells and stria vascularis. Furthermore, regulation of hearing ability by miRNA [49,50] has been reported previously and reviews suggest other epigenetic regulation involved in hearing and deafness disorders [51]. Specifically the enrichment of ephrin receptors and gene silencing ontologies in the gene-based association analysis of hearing ability might highlight new pathways involved in ARHI.

Replication of association with the same trait in an independent sample is considered a strong indication for true positive findings. This analysis was able to replicate genome-wide association with genes previously associated with hearing function. *FGF14*, *CSMD1* and *ARSG* have previously been reported in a GWAS meta-analysis of hearing function performed in samples of the G-EAR cohort [17,52]. As part of this work, *Arsg* and *Csmd1* were reported to show striking expression in sensory cells of mouse cochlea [52,53], supporting a function of the respective proteins in the inner ear.

To further validate our findings, association signals found in the pure-tone audiogram dataset were validated in independent subjects of the speech-in noise test sample and vice versa. On a gene-based level, *PANX1*, *LYST* and *WNT16* were found associated with ARHI in the pure-tone audiogram and this association validated in the speech-in-noise test sample. None of these genes had been linked to hearing ability with age previously, however, PANX1 is a gap-junction protein known to be expressed in the cochlea and spiral ganglion neurons [54]. *LYST* encodes a lysosomal protein trafficking regulator. *Lyst* knockout mice show lysosomal dysfunction and abnormal melanogenesis. *WNT16* has not yet been linked to hearing, however *Wnt4*, another member of the WNT family was essential for recovery of hair cells in the avian inner ear [55].

The problem of missing heritability [5] has been described for many common complex traits like diabetes and height. Experts have suggested a range of solutions to tackle this problem, including the use of more precise phenotypes to reduce heterogeneity, inclusion of rare genetic markers (MAF<1%) and structural genetic variants as well as study of more genetically diverse populations and larger sample sizes [5]. Many of these suggestions have been incorporated in these and previous GWASs of ARHI, including the use of isolated populations [17], selection of individuals with extreme phenotypes [14] and application of advanced phenotyping methods like principal component analysis. A limitation shared by all GWASs of hearing traits is the low to moderate sample size. Genome-wide association studies are based on the common disease-common variant hypothesis, which states that traits with a high prevalence in a population should be caused by genetic makers equally frequent in the population [56,57,58]. It has been argued though that genetic variants with a large effect on a trait could impact reproductive fitness and therefore be selected against by natural selection. This selection process would result in rare variants with high effect sizes and more common variants with moderate to low influence on the phenotype [58]. Due to the late age of onset and slow progression of ARHI [59], reduced reproductive fitness

and thus negative selection against ARHI is not to be expected. GWASs test for an association of common genetic variants (MAF≥0.05) with a selected phenotype. Significantly associated marker variants are expected to be in high LD with the causal underlying variant. However, common tagging SNP used in GWAS are only in weak LD with rare causal variants. Whole genome sequencing or exome sequencing would be required to test for associations between ARHI and rare variants. Without these datasets it is currently too early to comment on putative allele frequency distributions of causal variants in ARHI. Also, recent genetic studies on ARHI and hearing function suggest many more genes than originally suspected to be involved in hearing ability [1,6,8,14,17,60]. The multitude of genes involved will most likely result in low to moderate effect sizes for single variants, which could only be identified in samples much larger than the ones currently used in hearing research.

Despite all the strengths of this study, there were limitations that were beyond the scope of this project. Although a reasonable sample size was collected both for the ARHI (n=1028) and hearing function (n=1214) GWASs, the number of subjects did not give sufficient power to detect genome-wide significant associations. Furthermore, hearing ability for the TwinsUK sample was slightly better than expected from other epidemiological studies of similar age-range. A higher proportion of individuals with more extreme HL might have increased the effect size and therefore the chance to determine more significant genetic associations. Although many of the genes associated with hearing ability here were linked to hearing and deafness phenotypes, further research on these genes in model organisms would be desirable. The follow up of gene expression in the mouse cochlea reported by *Girotto et al* [52] seems a suitable method to verify putative function of associated genes in the inner ear. Lastly, participants used here were not unrelated as assumed for GWAS, still, previous associations studies in related individuals have proven the success and strength of this study design [26].

In conclusion, this study identified various associations of suggestive genome-wide significance. A gene-based association study as well as gene-enrichment analysis helped to verify findings from the discovery GWASs and thereby reduce the risk of false positive associations. It was concluded that ARHI and hearing function are likely to be determined by a multitude of common genetic variants with each showing low effects on the trait, like suggested previously [16]. To identify these common low effect variants larger sample sizes would be required, which can be achieved by combining data from different hearing and ARHI cohorts. Moreover, results from the gene enrichment analysis indicated that epigenetic processes might be involved in ARHI.

References

1. DeStefano AL, Gates GA, Heard-Costa N, Myers RH, Baldwin CT (2003) Genomewide linkage analysis to presbycusis in the Framingham Heart Study. Arch Otolaryngol Head Neck Surg 129: 285-289.

2. Garringer HJ, Pankratz ND, Nichols WC, Reed T (2006) Hearing Impairment Susceptibility in Elderly Men and the DFNA18 Locus. Arch Otolaryngol Head Neck Surg 132: 506-510.

3. Bonsch D, Scheer P, Neumann C, Lang-Roth R, Seifert E, et al. (2001) A novel locus for autosomal dominant, non-syndromic hearing impairment (DFNA18) maps to chromosome 3q22 immediately adjacent to the DM2 locus. Eur J Hum Genet 9: 165-170.

4. Huyghe JR, Van Laer L, Hendrickx JJ, Fransen E, Demeester K, et al. (2008) Genome-wide SNP-based linkage scan identifies a locus on 8q24 for an age-related hearing impairment trait. Am J Hum Genet 83: 401-407.

5. Manolio TA, Collins FS, Cox NJ, Goldstein DB, Hindorff LA, et al. (2009) Finding the missing heritability of complex diseases. Nature 461: 747-753.

6. Unal M, Tamer L, Dogruer ZN, Yildirim H, Vayisoglu Y, et al. (2005) N-acetyltransferase 2 gene polymorphism and presbycusis. Laryngoscope 115: 2238-2241.

7. Djordjevic N, Carrillo JA, Ueda N, Gervasini G, Fukasawa T, et al. (2010) N-acetyltransferase-2 (NAT2) Gene Polymorphisms and Enzyme Activity in Serbs: Unprecedented High Prevalence of Rapid Acetylators in a White Population. The Journal of Clinical Pharmacology.

8. Van Eyken E, Van Camp G, Fransen E, Topsakal V, Hendrickx JJ, et al. (2007) Contribution of the N-acetyltransferase 2 polymorphism NAT2*6A to age-related hearing impairment. J Med Genet 44: 570-578.

9. Van Laer L, Van Eyken E, Fransen E, Huyghe JR, Topsakal V, et al. (2008) The grainyhead like 2 gene (GRHL2), alias TFCP2L3, is associated with age-related hearing impairment. Hum Mol Genet 17: 159-169.

10. The International HapMap C (2005) A haplotype map of the human genome. Nature 437: 1299-1320.

11. Lander ES (1996) The New Genomics: Global Views of Biology. Science 274: 536-539.

12. Risch N, Merikangas K (1996) The Future of Genetic Studies of Complex Human Diseases. Science 273: 1516-1517.

13. Price AL, Patterson NJ, Plenge RM, Weinblatt ME, Shadick NA, et al. (2006) Principal components analysis corrects for stratification in genome-wide association studies. Nature Genetics 38: 904-909.

14. Friedman RA, Van Laer L, Huentelman MJ, Sheth SS, Van Eyken E, et al. (2009) GRM7 variants confer susceptibility to age-related hearing impairment. Hum Mol Genet 18: 785-796.

15. Van Laer L, Huyghe JR, Hannula S, Van Eyken E, Stephan DA, et al. (2010) A genome-wide association study for age-related hearing impairment in the Saami. Eur J Hum Genet 18: 685-693.

16. Fransen E, Bonneux S, Corneveaux JJ, Schrauwen I, Di Berardino F, et al. (2014) Genome-wide association analysis demonstrates the highly polygenic character of age-related hearing impairment. European Journal of Human Genetics.

17. Girotto G, Pirastu N, Sorice R, Biino G, Campbell H, et al. (2011) Hearing function and thresholds: a genome-wide association study in European isolated populations identifies new loci and pathways. Journal of Medical Genetics 48: 369-374.

18. Richards J, Rivadeneira F, Inouye M, Pastinen T, Soranzo N, et al. (2008) Bone mineral density, osteoporosis, and osteoporotic fractures: a genome-wide association study. The Lancet 371: 1505-1512.

19. Soranzo N, Rivadeneira F, Chinappen-Horsley U, Malkina I, Richards JB, et al. (2009) Meta-analysis of genome-wide scans for human adult stature identifies novel Loci and associations with measures of skeletal frame size. PLoS genetics 5: e1000445.

20. Kermani BG (2006) Artificial intelligence and global normalization methods for genotyping. Google Patents.

21. Teo YY, Inouye M, Small KS, Gwilliam R, Deloukas P, et al. (2007) A genotype calling algorithm for the Illumina BeadArray platform. Bioinformatics 23: 2741-2746.

22. Howie BN, Donnelly P, Marchini J (2009) A flexible and accurate genotype imputation method for the next generation of genome-wide association studies. PLoS genetics 5: e1000529.

23. Amin N, van Duijn CM, Aulchenko YS (2007) A genomic background based method for association analysis in related individuals. PLoS One 2: e1274.

24. Aulchenko YS, Ripke S, Isaacs A, van Duijn CM (2007) GenABEL: an R library for genome-wide association analysis. Bioinformatics 23: 1294-1296.

25. Aulchenko YS, de Koning D-J, Haley C (2007) Genomewide Rapid Association Using Mixed Model and Regression: A Fast and Simple Method For Genomewide Pedigree-Based Quantitative Trait Loci Association Analysis. Genetics 177: 577-585.

26. Chen W-M, Abecasis GR (2007) Family-Based Association Tests for Genomewide Association Scans. The American Journal of Human Genetics 81: 913-926.

27. R Core Team (2012) R: A language and environment for statistical computing. Vienna.

28. Turner S (2011) Annotated Manhattan plots and QQ plots for GWAS using R, Revisited

29. Liu JZ, Mcrae AF, Nyholt DR, Medland SE, Wray NR, et al. (2010) A versatile gene-based test for genome-wide association studies. The American Journal of Human Genetics 87: 139-145.

30. Gamazon ER, Zhang W, Konkashbaev A, Duan S, Kistner EO, et al. (2010) SCAN: SNP and copy number annotation. Bioinformatics 26: 259-262.

31. Kent WJ, Sugnet CW, Furey TS, Roskin KM, Pringle TH, et al. (2002) The Human Genome Browser at UCSC. Genome Research 12: 996-1006.

32. Fujita PA, Rhead B, Zweig AS, Hinrichs AS, Karolchik D, et al. (2010) The UCSC Genome Browser database: update 2011. Nucleic Acids Research.

33. Pruim RJ, Welch RP, Sanna S, Teslovich TM, Chines PS, et al. (2010) LocusZoom: regional visualization of genome-wide association scan results. Bioinformatics 26: 2336-2337.

34. Warde-Farley D, Donaldson SL, Comes O, Zuberi K, Badrawi R, et al. (2010) The GeneMANIA prediction server: biological network integration for gene prioritization and predicting gene function. Nucleic Acids Research 38: W214-W220.

35. Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society Series B (Methodological): 289-300.

36. Wolber LE, Steves CJ, Spector TD, Williams FMK (2012) Hearing Ability with Age in Northern European Women: A New Web-Based Approach to Genetic Studies. PLoS One 7: e35500.

37. Viljanen A, Era P, Kaprio J, Pyykkö I, Koskenvuo M, et al. (2007) Genetic and Environmental Influences on Hearing in Older Women. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences 62: 447-452.

38. Gates GA, Couropmitree NN, Myers RH (1999) Genetic associations in age-related hearing thresholds. Arch Otolaryngol Head Neck Surg 125: 654-659.

39. Karlsson KK, Harris JR, Svartengren M (1997) Description and primary results from an audiometric study of male twins. Ear Hear 18: 114-120.

40. Pillers D-AM, Kempton JB, Duncan NM, Pang J, Dwinnell SJ, et al. (2002) Hearing loss in the laminin-deficient dy mouse model of congenital muscular dystrophy. Molecular Genetics and Metabolism 76: 217-224.

41. Yamagata M, Weiner JA, Sanes JR (2002) Sidekicks: synaptic adhesion molecules that promote lamina-specific connectivity in the retina. Cell 110: 649-660.

42. Abbas L (2003) Synapse Formation: Let's Stick Together. Current Biology 13: R25-R27.

43. Costell M, Gustafsson E, Aszódi A, Mörgelin M, Bloch W, et al. (1999) Perlecan Maintains the Integrity of Cartilage and Some Basement Membranes. The Journal of Cell Biology 147: 1109-1122.

44. Hauge H, Fjelland KE, Sioud M, Aasheim H-C (2009) Evidence for the involvement of FAM110C protein in cell spreading and migration. Cellular Signalling 21: 1866-1873.

45. Schraders M, Lee K, Oostrik J, Huygen PLM, Ali G, et al. (2010) Homozygosity Mapping Reveals Mutations of GRXCR1 as a Cause of Autosomal-Recessive Nonsyndromic Hearing Impairment. The American Journal of Human Genetics 86: 138-147.

46. Odeh H, Hunker KL, Belyantseva IA, Azaiez H, Avenarius MR, et al. (2010) Mutations in Grxcr1 Are The Basis for Inner Ear Dysfunction in the Pirouette Mouse. The American Journal of Human Genetics 86: 148-160.

47. Lu B, Tigchelaar W, Ruifrok WP, van Gilst WH, de Boer RA, et al. (2012) DHRS7c, a novel cardiomyocyte-expressed gene that is down-regulated by adrenergic stimulation and in heart failure. European journal of heart failure 14: 5-13.

48. Defourny J, Poirrier A-L, Lallemend F, Mateo Sánchez S, Neef J, et al. (2013) Ephrin-A5/EphA4 signalling controls specific afferent targeting to cochlear hair cells. Nat Commun 4: 1438.

49. Lewis MA, Quint E, Glazier AM, Fuchs H, De Angelis MH, et al. (2009) An ENU-induced mutation of miR-96 associated with progressive hearing loss in mice. Nat Genet 41: 614-618.

50. Mencia A, Modamio-Hoybjor S, Redshaw N, Morin M, Mayo-Merino F, et al. (2009) Mutations in the seed region of human miR-96 are responsible for nonsyndromic progressive hearing loss. Nat Genet 41: 609-613.

51. Friedman L, Avraham K (2009) MicroRNAs and epigenetic regulation in the mammalian inner ear: implications for deafness. Mammalian Genome 20: 581-603.

52. Girotto G (2013) Genes and lifestyle in normal hearing function and age-related hearing loss [Doctoral Thesis]: Università degli studi di Trieste.

53. Girotto G, Vuckovic D, Buniello A, Lorente-Cánovas B, Lewis M, et al. (2014) Expression and Replication Studies to Identify New Candidate Genes Involved in Normal Hearing Function. PLoS One 9: e85352.

54. Tang W, Ahmad S, Shestopalov VI, Lin X (2008) Pannexins are new molecular candidates for assembling gap junctions in the cochlea. Neuroreport 19: 1253.

55. Alvarado DM, Hawkins RD, Bashiardes S, Veile RA, Ku Y-C, et al. (2011) An RNA Interference-Based Screen of Transcription Factor Genes Identifies Pathways Necessary for Sensory Regeneration in the Avian Inner Ear. The Journal of Neuroscience 31: 4535-4543.

56. Reich DE, Lander ES (2001) On the allelic spectrum of human disease. Trends in Genetics 17: 502-510.

57. Collins FS, Guyer MS, Chakravarti A (1997) Variations on a Theme: Cataloging Human DNA Sequence Variation. Science 278: 1580-1581.

58. Pritchard JK (2001) Are Rare Variants Responsible for Susceptibility to Complex Diseases? The American Journal of Human Genetics 69: 124-137.

59. Bedin E, Franzè A, Zadro C, Persico MG, Ciullo M, et al. (2009) Age-related hearing loss in four Italian genetic isolates: an epidemiological study. International Journal of Audiology 48: 465-472.

60. Van Laer L, Huyghe JR, Hannula S, Van Eyken E, Stephan DA, et al. (2010) A genome-wide association study for age-related hearing impairment in the Saami. European Journal of Human Genetics 18: 685-693.

Chapter 6: A GWAS meta-analysis discovers salt inducible kinase 3 as a new candidate gene for hearing function

Abstract

Hearing function and ARHI are known to be heritable, still genome-wide significant associations with these complex traits are rare, probably due to moderate sample sizes and corresponding lack of statistical power.

In this chapter, Genome-wide association study results from 8 different samples conducted by the G-EAR consortium and TwinsUK were combined in a meta-analysis of hearing function. Hearing function, measured by pure-tone audiometry, was assessed in 7 population samples of European ancestry (n=4591) and one sample from the Silk Road (n=348). Results from the pure-tone audiogram were summarised in 2 principal components adjusted for age and gender of participants. Genetic association studies were performed separately for each population, based on an additive genetic model adjusted for relatedness of subjects. Results from the studies were combined in a meta-analysis based on the direction of effect and significance of association weighted by sample size per population.

A single nucleotide polymorphism in intron 6 of the salt-inducible kinase 3 gene was genome-wide significantly associated ($p=3.69x10^{-8}$, Z-score=-5.505) with principal component 2, representing the slope of the audiogram. Immunohistochemistry of Sik3 in mouse cochlea revealed a striking expression profile of this protein in haircells, the spiral ganglion, stria vascularis and Reissner's membrane at different points in development. These results suggest a role of Sik3 in development of hearing ability and maintenance of the same throughout adulthood.

Introduction

As addressed in chapter 5, most GWAS of hearing phenotypes (i.e. hearing function or ARHI) lack sufficient sample sizes to reach statistical power to detect common variants with low effects on the trait under study. Genome-wide association studies (GWASs) of other common complex traits like height [1] or body mass index [2] have shown sample size to be positively correlated with the number of genome-wide significant associations [3]. The low sample sizes currently used in hearing traits are mainly

influenced by the high effort, specialised equipment, training and time required to collect pure-tone audiogram data. An alternative to separately collecting a large sample size for GWA is to combine the data from existing GWASs on the same trait in a meta-analysis.

The first GWAS meta-analysis of hearing function was conducted by the G-EAR consortium [4]. In this analysis the GWAS results from six isolated populations (n=3417, age range: 18-98 years, ~60% females) were combined in a meta-analysis. The meta-analysis was based on the significance of association and direction of effect (beta) and weighted by the sample size of the respective populations. The broad age range of this study made it difficult to refer to the phenotype as ARHI and was therefore described as adult hearing function. The use of isolated populations in this study increased the power to detect associations due to decreased genetic heterogeneity within the single populations. Hearing ability was measured in a standard pure-tone audiogram and summarized as pure-tone averages (over low, medium and high frequency ranges), pure-tone thresholds (250 Hz-8 kHz) and principal components (PC1-3). Several suggestive genome-wide associations ($p<10^{-7}$) were detected for genes including doublecortin-like kinase 1 (*DCLK1),* receptor-type tyrosine-protein phosphatase delta *(PTPRD),* metabotropic glutamate receptor type 8 *(GRM8)* and c-Maf inducing protein *(CMIP)*[4]. Associated genes with a p-value below 10^{-4} from all tested pure-tone audiogram phenotypes used were taken forward for a pathway analysis to detect shared pathways between candidate genes.

This study gives proof that higher sample sizes for studies of hearing traits can be reached under collaborative effort. Despite the lack of genome-wide significant associations, the increase in combined sample size resulted in several suggestive significant associations. Furthermore, combining GWA data from different cohorts allowed adjustment of individual biases applying to different cohorts (i.e. using different quality control thresholds), which would have been impossible if all samples had been pooled in one large GWAS of hearing function. However, the standards for phenotype collection have to be equal across all combined studies to prevent false positive associations.

Probably due to the lack of genome-wide significant associations, only few association signals for GWASs on ARHI or hearing function had previously been taken forward for functional analysis. Most studies limited their gene follow-up to literature search or pathway analyses of associated genes, but rarely investigated putative functions of genes in the cochlea *in vivo*. Expression data from the inner ear is scarce and rarely included in common expression databases. This gap of knowledge might be explained

by the low accessibility of human inner ear tissue pre mortem. Nevertheless, mouse models have been successfully applied to study human hearing disorders [5,6]. Mice serve as a perfect model organism for human hearing due to the high similarity in anatomy and physiology between murine and human inner ears. The temporal and spatial expression profile of candidate genes in the mouse cochlea can give essential information on the function and pathways a gene product might be involved in [5,6]. The G-EAR consortium has recently given a good example of how to follow up expression of genes identified in genome-wide association studies in mouse models [7].

Here we present the first genome-wide significant ($p<5x10^{-8}$) association with principal component (PC) 2, measuring the slope of the audiogram, determined in a GWAS meta-analysis of hearing function. The association signal (rs681524, $p=3.69x10^{-8}$, Z-score=-5.505) mapped to an intron of salt-inducible kinase 3. Immunohistochemistry of Sik3 in mouse cochlea revealed a striking expression of Sik3 in the inner ear at different developmental stages, supporting this gene as a novel candidate for hearing function.

Materials and Methods

Subjects

Subjects were recruited from two cohorts, the G-EAR consortium and TwinsUK. Samples from the G-EAR consortium originated from isolated communities throughout Italy (Carlantino, Cilento, Friuli Guilia and Talana), Croatia (Korcula and Split) and the Silk Road, while TwinsUK samples were recruited to study ageing traits in adult female twins residing in the United Kingdom. Samples from the G-EAR cohort and TwinsUK have been described elsewhere [4,8,9,10].

All individuals completed a hearing test and gave blood or saliva samples for DNA extraction. Volunteers were screened for a family history of hearing loss or previous ear diseases, which might cause conductive HL, by the mean of questionnaires. Subjects reporting either predisposition were excluded from further analysis. All research was conducted according to the ethical standards as defined by the Helsinki declaration. Studies were approved by the National Research Ethics service London-Westminster (REC reference number: 07/H0802/84), the Institutional Review Board of IRCCS-Burlo

Garofolo, Trieste, Italy and other involved members. Fully informed written consent was obtained from all participants prior to study conduction.

Phenotypes

All individuals participated in a standard pure-tone audiogram conducted according to local standards (i.e.[11]). Pure-tone thresholds [dB HL] measured for frequencies 250 Hz, 500 Hz, 1 kHz, 2 kHz, 4 kHz and 8 kHz were summarized in a principal component analysis. Principal components (PCs) 1 and 2 represented the overall threshold shift and slope of the audiogram, respectively, as described previously [12]. PC loadings for PC1 and PC2 were compared for direction between study groups and adjusted for age and gender in a linear regression and residuals taken. The resulting PC residuals were rank-transformed to normality separately for each sample. In case of PC loadings indicating a reverse direction for PC1 or PC2 in single populations, the direction was corrected by taking the reverse effect (beta) from the GWAS for meta-analysis, if necessary.

Genotyping and imputation

DNA extracted from individual samples was genotyped and imputed separately for each sample. Genotyping and Imputation for TwinsUK samples has been covered in detail in chapter 5. Subjects from the G-EAR cohort were genotyped using either the Illumina 370k or Affymetrix 500k array. Genotypes were called applying the appropriate software. Imputation was performed based on the HapMap Phase 2 CEU sample using Impute version 2 [13](TwinsUK) or the Markov Chain based haplotyper (MaCH 1.0)(G-EAR consortium)[14]. Single nucleotide polymorphisms (SNPs) with an imputation quality score below 0.4 (info in Impute vs 2) or 0.3 (Rsq in MaCH 1.0), respectively, were excluded for GWAS. Information on the genotyping platforms, imputation program and pre-imputation quality control thresholds used for each population are listed in supplementary Table 44.

Genome-wide association studies

Genome-wide association studies were performed separately for each community using R GenAbel [15] or ProbAbel [16] library. GWA was based on a linear mixed effect model assuming an additive genetic model and adjusting for putative relatedness of subjects [17,18,19], using the GRAMMAR option [18,19] available for mmScore. The exact method has been described in detail in chapter 5.

Meta-analysis in METAL

GWAS results for each population were combined in a meta-analysis of GWAS using METAL meta-analysis software [20]. The sample size scheme was chosen, in which GWAS results per SNP are combined by p-value and direction of effect weighted by the sample size of each population. This is particularly useful for meta-analysis of samples with large differences in sample size. In short, Z-scores were calculated for each GWAS sample per SNP based on the significance of association (p-value) and direction of effect (direction of beta). Z-scores per population were then combined to form an overall Z-score per SNP weighted by sample size of each population with GWAS results available for the respective SNP. Combined Z-scores can be both positive and negative, with higher absolute values indicating higher significance of association. The direction of the Z-score indicates whether the effect allele increases (Z-score >0) or decreases (Z-score<0) phenotypic measures [20]. SNPs with a minor allele frequency ≤0.01, a call rate ≤0.90 and/or significance of deviation from Hardy Weinberg equilibrium p(HWE) $\leq 10^{-6}$ were excluded from the meta-analysis.

Follow up of genome-wide significant association signals

Genome-wide significant association signals resulting from the GWAs meta-analysis were followed up in more detail using forest plots. The forest plot depicts the beta and corresponding 95% confidence interval per population studied in comparison to the combined beta. The total beta was calculated as the average of all betas per population weighted by the sample size of each population. This plot shows whether all populations show the same direction of effect for the respective generic marker and thereby helps to evaluate the validity and heterogeneity of the respective association. The percentage of total variation at rs681524 due to heterogeneity was calculated in form of I^2 [21]. Furthermore, genotyping cluster plots available for the TwinsUK sample were investigated for clarity of genotype separation and to support validity of our findings (Figure 51).

A regional LocusZoom [22] was generated to assess location of associated SNPs and correlation with nearby markers. Linkage disequilibrium (LD) surrounding (±500kb) genome-wide significant findings ($p \leq 5x10^{-8}$) were further investigated using the 1000 Genomes pilot 1, CEU population dataset (n=1092) [23] as extracted from the SNP Annotation and Proxy Search tool [24]. In addition, whole genome sequencing data for TwinsUK samples (n=2000) was available as part of the UK10K study. This data was used to newly impute the original genotyping data in n=5654 subjects from TwinsUK. The newly imputed TwinsUK sample was used to calculate LD structure in PLINK [25] investigating 400 neighbouring SNPs located in a 1 Mb window surrounding rs681524.

Imputation accuracy was measured as genotype concordance between imputed genotypes (used in the GWAS and meta-analysis) and newly available whole genome sequencing data at rs681524 in 2 subsamples from Carlantino (n=93) and Friuli Venezia Giulia (n=222). In addition, GWAS genotyping results from TwinsUK samples were compared with newly collected whole-genome sequencing data from the UK10K project (n=2000) at rs681524.

Furthermore, association between rs681524 and PC2 was validated in 503 females from the UK10K TwinsUK whole genotyping dataset with available hearing data applying a linear regression model (adjusted for age and twin relatedness).

Analysis of genome-wide significant associations by age groups

The effect of genome-wide significantly associated SNPs on hearing was analysed stratified by three age groups (>40 years, 40-60 years and >60 years) in all samples separately. These association analyses were conducted adjusted for relatedness between subjects, as described above. PC values used in this analysis were not adjusted for age of subjects and mean effect calculated for all samples per age group.

Immuno-histochemistry in mouse models

Expression analysis in mouse models was performed in collaboration with and under supervision of Professor Karen P. Steel and Dr. Annalisa Buniello. Facilities, equipment, mice and reagents were kindly provided by Professor Karen P. Steel. Wildtype C57Bl/6 mice homozygous for a spontaneous albino mutation were sacrificed at three different steps of development, the day of birth (P0), postnatal day 5 (P5) and at the age of 4 weeks (4w). Mouse heads were fixed in 10% formalin at 4ºC for 48 hours, followed by two washing steps of 30 min in phosphate buffered saline (PBS). For decalcification, 4w old specimens were kept in 10% ethylenediaminetetraacetic acid in PBS for 2.5 days at 4ºC. Following the decalcification, tissues were washed in PBS (2x 30 minutes at room temperature) and saline (2x2 hours at 4ºC). To dehydrate the samples, a dehydration chain of increasing ethanol concentration was used at 4ºC. Tissues were embedded overnight and mounted in paraffin wax the following morning. Serial 8 µm sections were mounted on glass slides and dried at 40ºC overnight. Immunohistochemistry was performed by the Ventana Discovery system (Ventana Medical Systems, Inc Illkirch, France) according to the manufacturer's instructions.

For each age, between 3 and 6 mice and multiple sections per animal were analysed and observations only reported if all samples showed similar labelling patterns. Two separate Sik3 antibodies were used and control sections were prepared omitting the primary antibody.

Antibodies

The following primary antibodies were used for immunohistochemistry experiments**:** primary Anti-SIK3 antibody (ab110987, Abcam, Cambridge, United Kingdom and as a control: LS-c120369 SIK3 Lifespan Bioscience, Seattle, USA) at 1:50 (P0, P5) or 1:40 (4w) concentration; anti-peripherin antibody (ab4666, Abcam, Cambridge, United Kingdom) at a 1:100 concentration; anti-TUJ1 antibody (Covance, New Jersey, US) at a 1:100 concentration; anti-GFAP antibody (ab53554, Abcam, Cambridge, United Kingdom) at a 1:50 concentration. The secondary antibody, biotin conjugated donkey anti-rabbit (711-065-152), was purchased from Jackson ImmunoResearch (West Grove, PA, USA). Images of the antibody stained sections were taken using a Zeiss Axioskop MOT light microscope. Image processing was performed in Adobe Photoshop CS5.

Confocal microscopy

Confocal Microscopy was kindly performed by Beatriz Lorente-Canovas with facilities, reagents, mice and equipment provided by Professor Karen P. Steel. Heads from 5 days old mice (P5) were bisected and inner ears plus bone were removed from the skull and then fixed in 4% paraformaldehyde for 2 hours at room temperature. Subsequently specimens were fine dissected in PBS, then washed and permeabilized in 1% PBS/Triton-X-100 (PBT) and blocked with 10% sheep serum. Then, they were incubated with the primary antibody, rabbit polyclonal against Sik3 (ab110987, Abcam, Cambridge, United Kingdom, dilution 1:200) overnight at 4°C. After washes with PBT, samples were incubated with anti-rabbit Alexa Fluor 488 secondary antibody (Invitrogen, anti-rabbit, diluted 1:500) and rhodamine/phalloidin (Invitrogen, diluted 1:100). Samples were mounted in Prolong Gold Antifading reagent (Invitrogen). Images were acquired on a LSM 510 Meta confocal microscope (Zeiss, Welwyn Garden City). Images were processed using Adobe Photoshop CS5.

Results

Subjects

Subjects were recruited from 8 different samples: 6 isolated community samples from Italy (Carlantino; Friuli Venezia Guilia; Cilento; Talana) and Croatia (Korcula; Split), one sample recruited from 5 different countries along the Silk Road and a twin cohort from the United Kingdom (TwinsUK). Sample sizes for the different samples ranged from 280 to 1097 individuals. In total, 4939 subjects were included in the GWAS meta-analysis with a preponderance of female volunteers (54.60%- 100% females). Age of participants ranged from 18 to 98 years with a mean age of 41.59- 61.06 years. The characteristics of the different study samples are summarised in Table 36.

Table 36 Characteristics of subjects by community

sample	country	n	gender (%females)	age (years)	
				range	mean (SD)
Carlantino	Italy	280	56.87%	18-89	53.29 (18.1)
Friuli Venezia Guilia	Italy	1097	60.19%	18-89	51.47 (16.3)
Korcula	Croatia	804	63.30%	18-98	56.30 (13.7)
Split	Croatia	497	56.00%	18-79	49.00 (14.6)
Cilento	Italy	421	56.67%	18-91	56.30 (17.6)
Talana	Italy	470	59.00%	18-92	50.82 (18.5)
Silk Road	Azerbaijan Giorgia Kazakhstan Tajikistan Uzbekistan	348	54.60%	18-82	41.59 (15.5)
Twins UK	United Kingdom	1022	100%	29-86	61.06 (9.1)
Total	**-**	**4939**	**-**	**18-98**	**-**

Samples included in the GWAS meta-analysis of hearing function are listed by their country of origin, the number of individuals per sample (n), gender distribution expressed as percentage of female subjects (% female) and age of participants (measured as age range and mean age with standard deviation from the mean (SD).

GWAS Meta-analysis

After quality control, as described in supplementary Table 44 and under Materials and Methods, >2.3 million SNPs were tested for association in the GWAS meta-analysis of hearing function. Only one SNP on chromosome 11 (rs681524, $p=3.69x10^{-8}$, Z-Score= -5.505) showed genome-wide significant ($p<5x10^{-8}$) association with PC2 (Table 46).

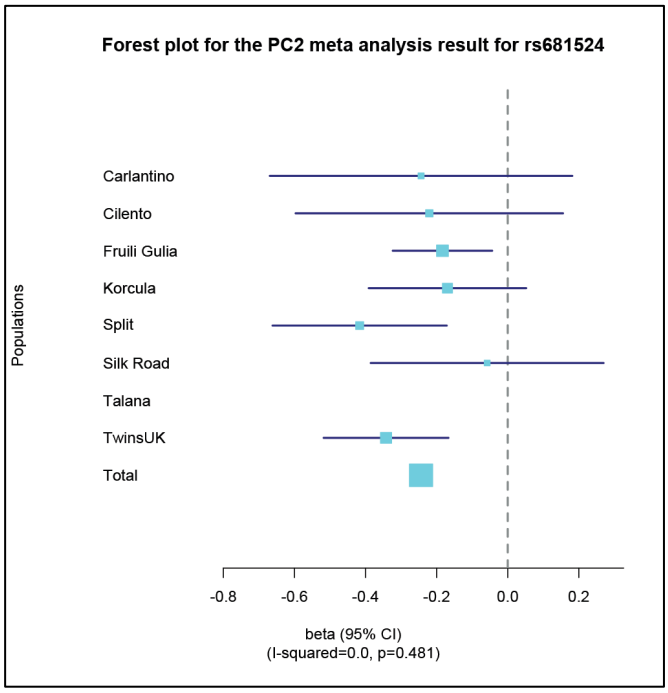


Figure 37 Forest plot of the PC2 GWAS meta-analysis findings at rs681524

The forest plot shows the direction of effect for each population at rs681524 compared to the combined effect. The direction of effect is measured as beta (light blue boxes) and displayed with corresponding 95% confidence interval (95% CI)(dark blue lines). The size of each box reflects the relative sample size per population. The combined direction of effect is displayed as total light blue box. Heterogeneity (I-squared) between study populations explained 0% of variation at rs681524 (p=0.481).

Table 37 GWA meta-analysis results at rs681524

population	n	MAF	beta	se	p-value	Z-Score
Carlantino	280	0.057	-0.2438	0.2215	0.2710	-1.101
Cilento	419	0.075	-0.2206	0.1958	0.2599	-1.127
Friuli Giulia	1097	0.080	-0.1836	0.0728	0.0117	-2.522
Korcula	794	0.064	-0.1695	0.1153	0.1415	-1.470
Split	497	0.087	-0.4166	0.1276	0.0011	-3.265
Silk Road	255	0.039	-0.0579	0.1705	0.7342	-0.340
Talana	-	-	-	-	-	-
TwinsUK	980	0.065	-0.3423	0.0914	1.8E-04	-3.745
total	**4322**	**-**	**-0.2439**	**-**	**3.69E-08**	**-5.505**

GWA meta-analysis results are presented as GWAS results per population (GWA results for the population of Talana were not available at this SNP) and total meta-analysis results. Each population is described by the number of samples (n) with available data at this SNP, the minor allele frequency (MAF), the direction of effect (beta) and corresponding standard error (se), the significance of association (p-value) and the Z-score calculated in Metal. The total Z-score is a combined value of the Z-scores per population weighted by sample size. These results are further depicted in the Forest plot (Figure 37).

Imputation accuracy measured as genotype concordance between imputation and whole genome sequencing results at rs681524 in 2 subsamples from Carlantino (n=93) and Friuli Venezia Giulia (n=222) determined high accuracies of 0.97 and 0.94, respectively. In addition, GWAS genotyping results from TwinsUK samples were compared with newly collected whole-genome sequencing data from the UK10K project (n=2000), confirming a high genotyping accuracy of 0.99.

The LocusZoom (Figure 38) showed that the association of PC2 with Sik3 was limited to SNP rs681524 at this locus on chromosome 11. Heterogeneity between study samples accounted for 0% of the variation at rs681524 (I^2=0.0, p=0.481). This single SNP association was likely influenced by the exceptionally low LD ($R^2 \leq 0.28$) of the reference SNP rs681524 with nearby genetic makers (± 400 kb) as shown in Figure 38. LD structure in the Locus Zoom was based on the HapMap Phase 2 CEU panel.

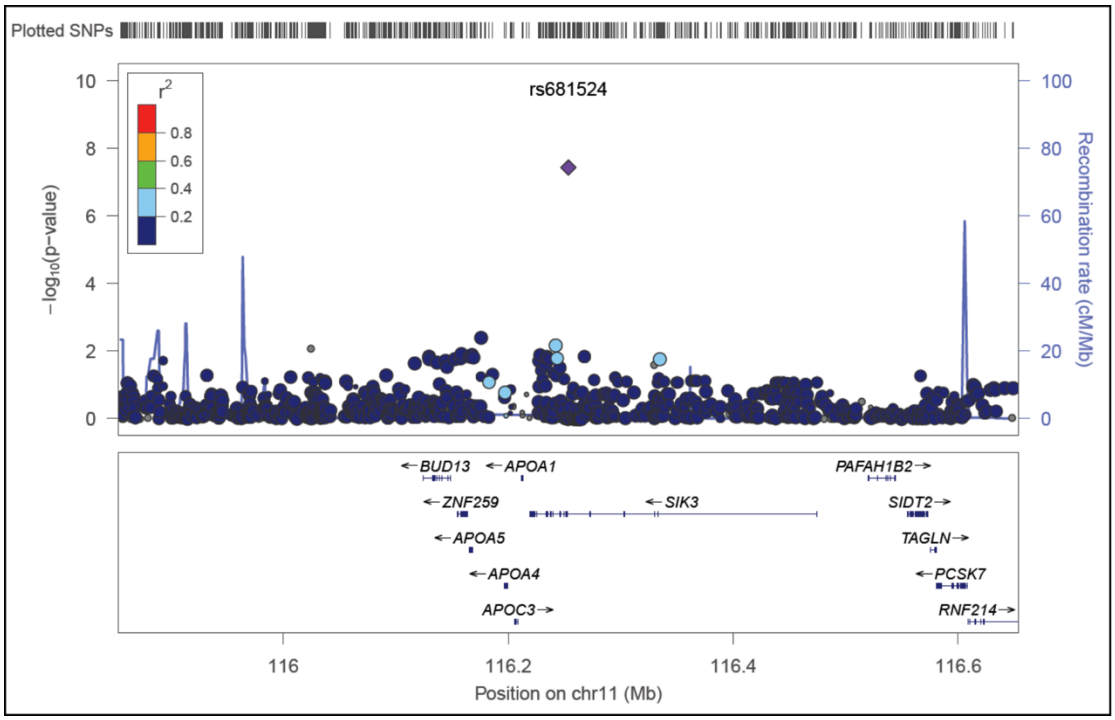


Figure 38 LocusZoom of GWAS meta-analysis results for PC2 at *SIK3*

The LocusZoom depicts the location of genetic markers versus their significance of association with PC2 values. Significance of association is measured as the negative logarithm of the p-value. Genes located in the area 400kb up-and downstream of rs681524 (violet diamond) are displayed below the x-axis. The colour of each genetic marker indicates its correlation with rs681524. A legend for the correlation colour scheme is shown in the upper left corner. Recombination rate is highlighted as light blue peaks.

Further information concerning LD structure at this locus was sought in two independent samples: the 1000 Genomes pilot dataset (n=1092) [23] and a newly imputed TwinsUK genotyping sample (n=5654). Both samples confirmed the low LD surrounding rs681524. While five SNPs, four of which were located in introns of *SIK3,* showed moderate LD (R^2 ≥0.8) with rs681524 in the 1000 Genomes sample, LD calculations using a newly imputed TwinsUK sample were uncommonly low for rs681524 (R^2≤0.28).

A linear regression between hearing PC2 and genotype at rs681524 (adjusted for age and twin relatedness) performed in a subset of the newly imputed TwinsUK sample (n=503 females) supported the negative association between PC2 and rs681524 (p=0.010, beta± se=-0.33 ± 0.13).

Association analysis with rs681524 by age groups

As the interest of this project lies originally in age-related hearing impairment, subgroup analysis of rs681524 by age groups was performed. Association analyses were conducted for under 40 years, 40 to 60 years and older than 60 years. The protective

effect of the C allele of rs681524 was detectable in all three age groups with a mean effect ranging from -0.117 to -0.386 (Table 38). The largest effect could be detected in the youngest samples (>40 years) although this was the smallest sample.

Table 38 Association of rs681524 with hearing PC2 stratified by age groups

	<40 years			40-60 years			>60 years		
Population	**n**	**beta**	**se**	**n**	**beta**	**se**	**n**	**beta**	**se**
Carlantino	88	-0.448	0.613	87	0.468	0.599	105	-0.412	0.403
Cilento	82	-0.956	0.385	150	-0.675	0.282	188	0.105	0.274
Friuli Venezia Giulia	295	0.124	0.116	424	-0.518	0.170	378	-0.258	0.210
Korcula	103	0.089	0.306	399	0.304	0.183	292	0.349	0.243
Split	138	-0.307	0.257	232	-0.595	0.218	127	-0.263	0.272
Silk Road	115	-0.210	0.424	104	0.253	0.428	36	-0.027	0.691
Talana	NA	NA	NA	NA	NA	NA	NA	NA	NA
TwinsUK	19	-0.993	0.671	372	-0.356	0.162	589	-0.314	0.113
Mean effect	120	-0.386	0.396	253	-0.160	0.292	245	-0.117	0.315

The effect of the C allele at rs681524 on hearing PC2 was analysed in three age groups (>40 years, 40-60 years and >60 years) in all samples separately. For each association the sample size (n), effect size (beta) and standard error of the effect size (se) are given. A mean effect was calculated for all samples per age group (beta, se).

Sik3 expression in the mouse cochlea

To investigate putative functions of *Sik3* in the inner ear, the expression pattern of this gene was investigated in mouse cochlea at different developmental stages (P0, P5 and 4w). Two separate Sik3 antibodies were used and both gave identical labelling, supporting the validity of the expression patterns detected. Control sections showed no noticeable labelling. The results of the expression analysis are summarised in Figure 39.

At the day of birth (P0), *Sik3* expression was detected at the top of both inner and outer hair cells (HCs), in distinct cells of the spiral ganglion (SG), in perilymph facing cells of the Reissner's membrane and in cells surrounding blood vessels in the stria vascularis. The cells expressing Sik3 in the stria vascularis have recently been identified as macrophage-like melanocytes, which are critical for integrity of the intrastrial fluid-blood barrier [26,27]. The same expression pattern could be detected 5 days postnatal (P5). However, at 4 weeks of age (4w) *Sik3* expression was limited to the SG, Reissner's membrane and the stria vascularis. No expression could be detected in the HCs at this advanced stage of development (Figure 39).

Sik3 expression in hair cells of the cochlea

To further investigate the exact location of Sik3 expression in the HCs at early developmental stages, confocal microscopy was used. These experiments were conducted by Beatriz Lorente-Canovas and kindly provided for inclusion in this thesis. Sik3 expression in the inner and outer HCs was compared to phalloidin expression. Phalloidin binds to actin filaments and therefore serves as marker for stereocilia, which are rich in actin. In comparison to phalloidin, Sik3 seemed to be primarily expressed at the top of the HCs but did not co-localise with phalloidin expression at the stereocilia (Figure 39, K-M).

Sik3 expression in cells of the spiral ganglion

In Figure 39 it was shown that Sik3 was expressed in distinct cells of the SG at all three tested stages of development (P0, P5 and 4w). To determine the exact cell type, which expresses Sik3 in the SG, Sik3 expression was compared to the expression pattern of known markers for type 1 and type 2 SG neurons (Beta-Tubulin and Peripherin) as well as glial cells (GFAP) in adjacent sections of the SG. Beta-Tubulin is expressed in both type 1 and type 2 SGs, whereas Peripherin is expressed in type 2 SG neurons only. Sik3 expression did not co-localize with either Peripherin or Beta-Tubulin (Figure 40, A-F) and thus not expressed in neurons. Further comparison of Sik3 expression with GFAP in adjacent sections showed a partial overlap in cells expressing Sik3 and GFAP, however the overlap was not complete (Figure 40, G-J).

Figure 39 Sik3 is expressed in various cells of the cochlea (next page)

Mouse cochlear sections were stained with primary Sik3 antibody (brown) and counterstained with hematoxylin (blue). **A, B, E, F**: At the day of birth (P0), Sik3 expression was detected in the apex of inner and outer hair cells (**B**, red arrows), in the perilymph facing layer of the Reissner's membrane (**F**, grey arrows), near blood vessels of the intermediate layer of the stria vascularis (**F**, black arrows), as well as in cells of the spiral ganglion (**E**, white arrows) and cells surrounding the ganglion. **C, D, G, H**: At 5 days postnatal (P5), Sik3 expression was found in the hair cells (**D**, red arrows) and small cells of the spiral ganglion (**G**, white arrows). **I, J:** At 4 weeks postnatal, Sik3 expression remained in the spiral ganglion (**J**, white arrows), Reissner's membrane and stria vascularis, but could not be detected in the apex of the hair cells (**I**). **K, L, M**: Convocal imaging of Sik3 expression in the stereocilia at 5 days postnatal (**L**, green), compared to Phalloidin expression (**K**, red), showed that Sik3 was expressed in the region around the base of the stereocilia (**M**, merged image of **K** and **L**). IHCs= Inner hair cells; OHCs= Outer hair cells. Scale~ bars: **A**, **C, I**: 50µm; **B**, **D**, **E**, **F**, **G**, **H**, **J**: 10 µm; **K**, **L**, **M**: 5 µm.

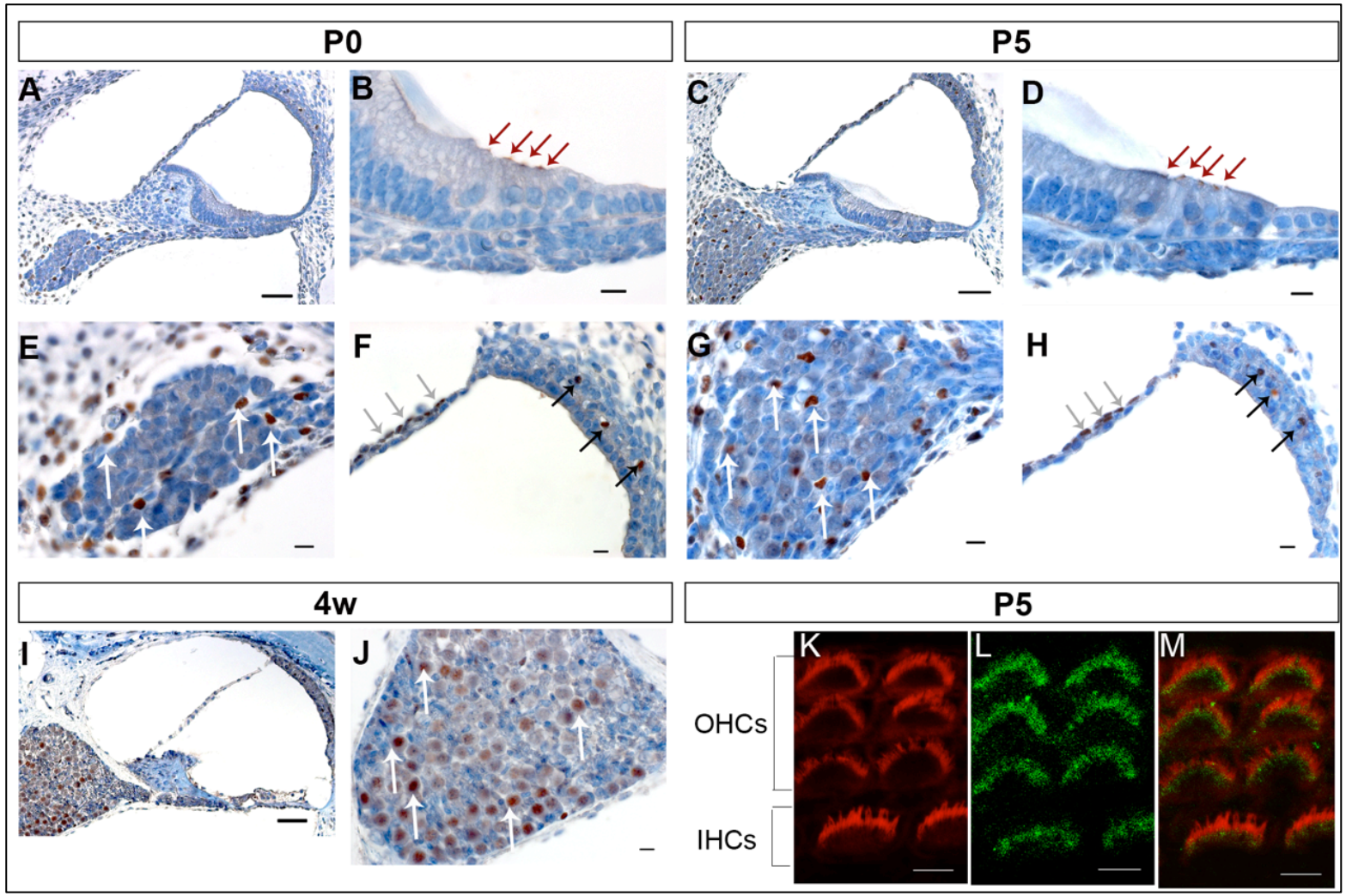


Figure 39 Sik3 is expressed in various cells of the cochlea

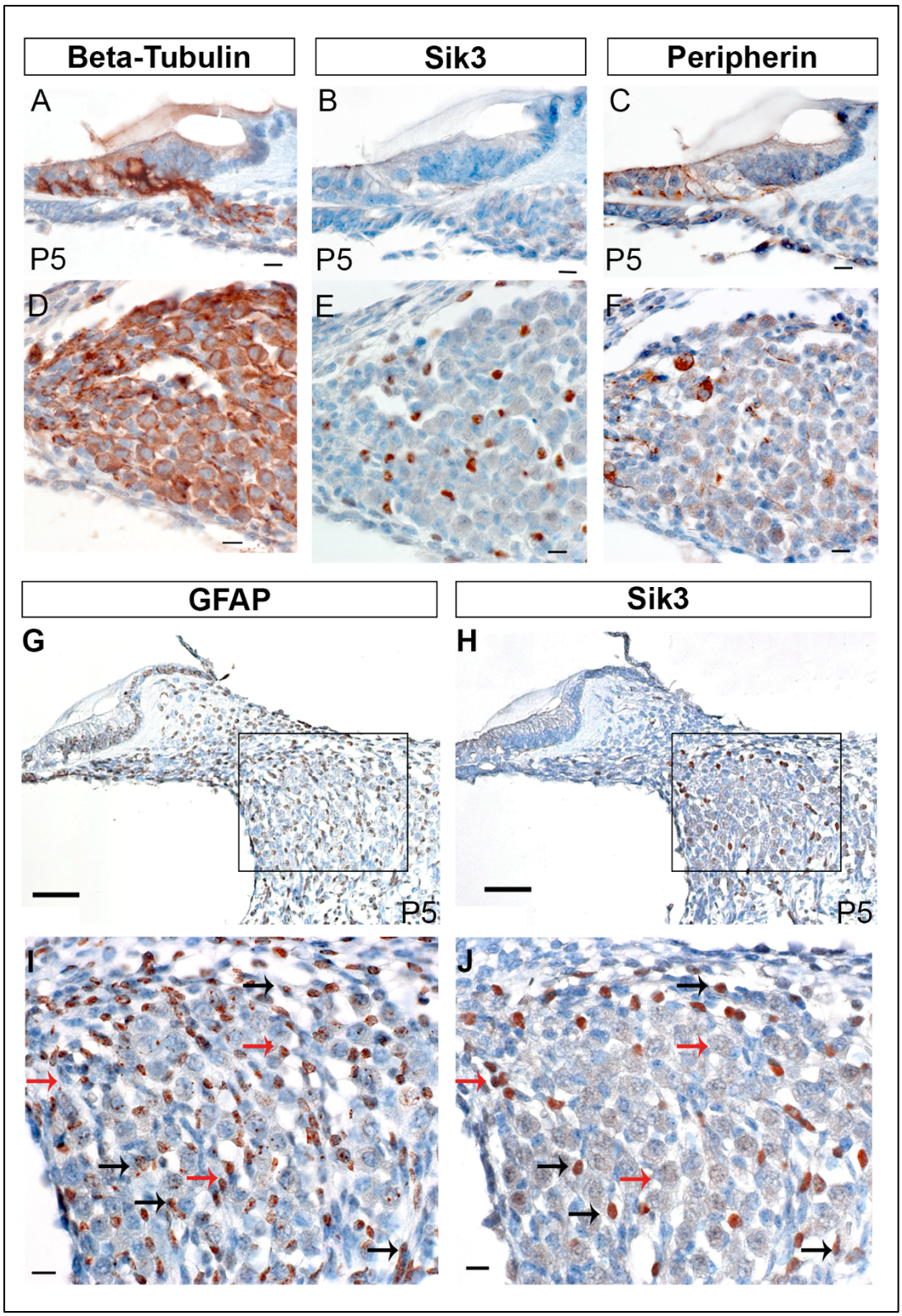


Figure 40 Sik3 expression in the SG is limited to small non-neuronal cells

To identify the cell type expressing Sik3 in the spiral ganglion Sik3 expression was compared to Beta-Tubulin **(A, D**), Peripherin (**C**, **F**) and GFAP (**G**, **I**) expression in adjacent sections. Beta-Tubulin is expressed in both Type 1 and 2 spiral ganglion neurons, while Peripherin expression is limited to type 1 spiral ganglion neurons. Sik3 expression (**B**, **E**) did not coincide with either Beta-Tubulin (**A**, **D**) or Peripherin expression (**C**, **F**), but seemed to be expressed in smaller cells interspersed between the neurons. To determine whether Sik3 could be expressed in glial cells, Sik3 expression (**H**, **J**) was compared to GFAP expression (G, I) in adjacent sections. Sik3

and GFAP expression overlapped partially. Sik3 expression coincided with GFAP expression in some cells (black arrows), but was absent in other glial cells (red arrows). All sections were prepared from mice at 5 days postnatal (P5). Expression of each antibody is indicated by a brown signal. Scale~ bars: **A**, **B**, **C**, **D**, **E**, **F**, **I**, **J**: 10 µm; G, H: 50 µm.

Discussion

Here we described the first genome-wide significant association with hearing function identified in a large GWAS meta-analysis (n=4939) of this trait. A SNP in intron 6 of the salt-inducible kinase 3 gene (*SIK3*) was genome-wide significantly associated (Z-score= -5.5, $p=3.69x10^{-8}$) with PC2, representing the slope of the audiogram. The LocusZoom of the described association signal on chr 11 did not support the validity of this finding due to a lack of nearby associated genetic markers. The concern that the association with rs681524 might be a false positive finding was addressed accordingly.

A forest plot was created (Figure 37), which highlighted the direction and strength of effect observed for the C allele at rs681524. The negative effect was consistent for all available populations and nominally significant in three of the eight samples. This association could not be explained by heterogeneity between samples ($I^2=0.0$, p=0.481). The exceptionally low LD structure surrounding rs681524 as seen in the HapMap CEU panel (

Figure 38), was confirmed in two independent panels, one from the 1000 Genomes project and a newly imputed TwinsUK sample.

As this SNP was genotyped in one sample (TwinsUK) and imputed in the others, the imputation accuracy of this SNP was investigated. SNP rs681524 was successfully imputed, passing imputation quality thresholds in 6 samples. In addition, imputation accuracy was assessed in two subsets from Carlantino and Friuli Venezia Giulia, which had additional whole genome sequencing data available. Imputation accuracy as analysed in these two subsets was high with values of 0.94 and 0.97. To exclude genotyping errors, the genotyping cluster plot for rs681524 (in TwinsUK) was examined (Figure 51), showing clear separation of alleles at this SNP. In addition, we were able to confirm a high genotyping accuracy of 0.99 for the TwinsUK sample.

Further, a regression analysis between hearing PC2 and rs681524 in a subset (n=503) of the newly imputed TwinsUK sample confirmed our original finding (p=0.010, beta± se=-0.33 ± 0.13). The exceptionally low LD reported for this SNP would rather be

expected for rare variants (MAF<5%). MAF at rs681524 was consistently low (MAF≤ 8%) in all presented populations but not rare.

Further to this evidence, *salt-inducible kinase 1*, another member of the family of salt-inducible kinases had been shown to be expressed in the human vestibule [28]. In summary, *Sik3* was assumed to be a suitable candidate gene for hearing function and thus selected for further expression studies in the cochlea. Due to the low accessibility of human cochlea in healthy individuals, the expression study was performed in cochlea of mice, whose inner ear anatomy and physiology is highly similar to that of humans.

Immunohistochemistry of *Sik3* in mouse cochlea was performed at three developmental stages (P0, P5 and 4w) to study the expression pattern of *Sik3* over time. *Sik3* showed a striking expression at the apex of the HCs, in non-neuronal cells of the SG, the perilymph facing layer of Reissner's membrane and in macrophage-like melanocytes in the stria vascularis. While expression in HCs was limited to early developmental stages (P0 and P5), expression in the other described structures lasted during early development up to adulthood (P0, P5, 4w). It was therefore assumed that *Sik3* serves a developmental function in HCs showing expression during early development whereas *Sik3* expression at other cochlea structures is more important for maintenance of the latter and thereby remains expressed during development and adulthood. Bright field microscopy was not sufficient to determine the exact location of *Sik3* expression in HCs at P0 and P5, therefore confocal microscopy was applied. Confocal microscopy revealed that *Sik3* was expressed at the apex of the HCs at the base of the stereocilia rather than in stereocilia themselves.

The striking expression pattern of *Sik3* in the SG was investigated in more detail by comparing *Sik3* expression of markers for Type 1 and 2 SG neurons and glial cells in adjacent sections. It was obvious that neither Beta-tubulin nor Peripherin (molecular markers for Type 1 and/or Type 2 SG neurons) were expressed in the same cells of the SG as *Sik3*. Having ruled out *Sik3* expression in SG neurons, we investigated whether *Sik3* might be expressed in glial cells interspersed between neurons. Gfap is a marker for glial cells in the SG and its expression was thus chosen to be compared to *Sik3*. A partial overlap between cells expressing *Sik3* and *Gfap* could be detected, however, it should be mentioned that co-localisation of Sik3 and Gfap was not complete for all cells. In addition, Sik3 appeared to be expressed in the cell nucleus, while Gfap was

expressed in cytoplasmic structures. It was thus concluded that *Sik3* is expressed in small non-neuronal cells in the SG, which might be an undefined subset of glial cells.

Salt-inducible kinases (SIKs) are a sub family of the AMP activated serine/threonine protein kinases. Three different SIKs have been identified, with Sik1 and Sik2 being expressed in adrenal and adipose tissue, respectively [29]. Initially, Sik3 was reported to be expressed ubiquitously. All three proteins contain a kinase, a sucrose non-fermenting homology and a phosphorylation domain [29]. SIK3 is with 1263 reported amino acids the largest protein of the three SIKs [29]. Both Sik1 and Sik3 control histone deacetylases via phosphorylation and nuclear export [30,31,32]. In addition, SIKs have recently been shown to regulate the formation of regulatory macrophages by phosphorylation of the CREB-regulated transcriptional coactivator 3 [33]. Analysis of Sik3 deficient mice revealed that Sik3 is essential for chondrocyte hypertrophy [31] and might be involved in regulation of lipid storage and cholesterol bile acid homoeostasis [34]. Mice with a disrupted Sik3 sequence ($Sik3^{-/-}$) present with dwarfism, skeletal abnormalities and malnourishment [31,34], however, details on their hearing ability have not been reported. In addition, 90% of $Sik3^{-/-}$ pups die on the first day after birth [31]. Interestingly, Sik3, was capable of binding histone deacetylase 4 (HDAC4) and thereby anchored it to the cell cytoplasm [31]. HDAC4 is known to transcriptionally repress myocyte-specific enhancer factor 2 when in the nucleus. Sik3 anchoring HDAC4 to the cytoplasm thus relieves this transcriptional repression [31]. These reports support the function of *Sik3* as an important regulator involved in various pathways.

The striking expression of Sik3 in cells essential for hearing underlines its putative role in hearing. It has been shown that mutation or down regulation of genes expressed in similar structures of the cochlea resulted in hearing loss or deafness. It would be of high interest to test the hearing ability of $Sik3^{-/-}$ mice, to determine whether Sik3 is essential for hearing.

A subset of the samples presented here had been used previously in a GWAS meta-analysis of hearing function [4]. The current analysis included two further GWAS samples, a female twin sample from the United Kingdom (TwinsUK) and a population sample collected from countries situated along the Silk Road (Azerbijan; Giorgia; Kazakistan; Tajikistan; Uzbekistan) [8]. By increasing the sample size, and therefore statistical power, it was hoped to determine associations with hearing of genome-wide significance. In comparison to the previously performed meta-analysis, the overlap of

significant associations was limited. The association of rs681524 has not been reported in the previous study, possibly because this association was strongly supported by the newly added TwinsUK GWAS (Table 37, p(TwinsUK)=1.8x10^{-4}). In addition, the previous study reported suggestive significant associations with metabotropic glutamate receptor type 8 (PC1, p-value=3.22x10^{-7}); the PBX/knotted homeobox 2 gene (PC2, p-value=2.86x10^{-7}) and phosphatidic acid phosphatase type 2D (PC3, p-value=2.32x10^{-7}) [4]. The association between PC1 and SNPs in the vicinity of *GRM8* was confirmed in the present study (Table 45; rs2687481, p=1.07x10^{-7}), while the association between PC1 and intronic SNPs in Immunoresponsive 1 homolog (*IRG1*, rs589636, p=6.61x10^{-8}) as reported here was novel. The association between PC2 and Carboxypeptidase A6 (Table 46; *CPA6,* rs1393902, p=3.07x10^{-7}) presents a further novel finding. The difference in association findings might reflect the addition of two samples of diverging ethnic origin (TwinsUK and Silk Road) to a relatively homogenous sample of southern European background. The change in findings might indicate that the presented samples had differing contribution of genomic variants to hearing function.

Despite the strengths of this study, there were details that were beyond the scope of this chapter. Although we were able to follow up expression of *Sik3* in murine cochlea, functional follow up in humans awaits confirmation. Furthermore, subjects selected for this study were of a wide age range and gender distribution, which differed between populations. To adjust for this difference in age and gender, PCs were adjusted for age at hearing test and gender previous to analysis. In addition, populations studied here were of different ethnic background, which increases the risk of population stratification. To account for this, population sub-structure was controlled for in each GWAS separately per population. A comparison of three samples of related subjects has shown this method to be similar in power and type 1 error to the more commonly used Genomic Control [18]. The difference in ethnicity should have also been considered when estimating LD pattern surrounding association results. LD pattern was initially followed up using the HapMap Phase 2 CEU panel, which might not represent LD pattern in some of the isolated populations presented here. To address this possible source of error, allele frequencies for the respective SNP were compared in data from the Human Genome Diversity project [35] and found similar across the selected populations. SNP rs681524 was genotyped in one of the 8 presented populations and successfully imputed in 6 further samples. Although examination of the cluster plots in TwinsUK showed a clear separation of genotypes, direct genotyping information for the remaining 7 samples would have strengthened our finding. It might be argued that the threshold for genome-wide significance should have been corrected

for the two, by definition, independent PCs investigated here. However, all three PCs were determined from the same pure-tone audiogram and thereby not truly independent. Although follow up of expression patterns gave further insight into putative functions of Sik3, no mechanistic links could be determined without further information gained in sik3 knockout mice.

Conclusion

In this chapter we presented the first genome-wide significant association between PC2, representing the slope of the audiogram and salt-inducible kinase 3. An expression study of *sik3* in the cochlea revealed striking expression patterns in various cells essential for hearing ability. *SIK3* is therefore proposed as a novel candidate gene for hearing function, which might be crucial for development of HCs and maintenance of hearing function in adults. Future analyses of *Sik3* in hearing should focus on the expression pattern of *SIK3* in human cochlea and hearing ability of mice with a disrupted *Sik3* sequence.

References

1. Visscher PM (2008) Sizing up human height variation. Nature Genetics 40: 489-490.
2. Speliotes EK, Willer CJ, Berndt SI, Monda KL, Thorleifsson G, et al. (2010) Association analyses of 249,796 individuals reveal 18 new loci associated with body mass index. Nature Genetics 42: 937-948.
3. Visscher Peter M, Brown Matthew A, McCarthy Mark I, Yang J (2012) Five Years of GWAS Discovery. The American Journal of Human Genetics 90: 7-24.
4. Girotto G, Pirastu N, Sorice R, Biino G, Campbell H, et al. (2011) Hearing function and thresholds: a genome-wide association study in European isolated populations identifies new loci and pathways. Journal of Medical Genetics 48: 369-374.
5. Avraham KB (2003) Mouse Models for Deafness: Lessons for the Human Inner Ear and Hearing Loss. Ear and Hearing 24: 332-341.
6. Ohlemiller KK (2006) Contributions of mouse models to understanding of age- and noise-related hearing loss. Brain Research 1091: 89-102.
7. Girotto G, Vuckovic D, Buniello A, Lorente-Cánovas B, Lewis M, et al. (2014) Expression and Replication Studies to Identify New Candidate Genes Involved in Normal Hearing Function. PLoS One 9: e85352.

8. Girotto G, Pirastu N, Gasparini A, D'Adamo P, Gasparini P (2011) Frequency of hearing loss in a series of rural communities of five developing countries located along the Silk Road. Audiological Medicine 9: 135-140.

9. Bedin E, Franzè A, Zadro C, Persico MG, Ciullo M, et al. (2009) Age-related hearing loss in four Italian genetic isolates: an epidemiological study. International Journal of Audiology 48: 465-472.

10. Moayyeri A, Hammond CJ, Valdes AM, Spector TD (2012) Cohort Profile: TwinsUK and Healthy Ageing Twin Study. International Journal of Epidemiology.

11. BSA (2011) Recommended Procedure for Pure-tone air-conduction and bone-conduction threshold audiometry with and without masking. Reading: British Society of Audiology.

12. Huyghe JR, Van Laer L, Hendrickx JJ, Fransen E, Demeester K, et al. (2008) Genome-wide SNP-based linkage scan identifies a locus on 8q24 for an age-related hearing impairment trait. Am J Hum Genet 83: 401-407.

13. Howie BN, Donnelly P, Marchini J (2009) A Flexible and Accurate Genotype Imputation Method for the Next Generation of Genome-Wide Association Studies. PLoS Genet 5: e1000529.

14. Li Y, Willer CJ, Ding J, Scheet P, Abecasis GR (2010) MaCH: using sequence and genotype data to estimate haplotypes and unobserved genotypes. Genetic Epidemiology 34: 816-834.

15. Aulchenko YS, Ripke S, Isaacs A, van Duijn CM (2007) GenABEL: an R library for genome-wide association analysis. Bioinformatics 23: 1294-1296.

16. Aulchenko YS, Struchalin MV, van Duijn CM (2010) ProbABEL package for genome-wide association analysis of imputed data. BMC Bioinformatics 11: 134.

17. Chen W-M, Abecasis GR (2007) Family-Based Association Tests for Genomewide Association Scans. The American Journal of Human Genetics 81: 913-926.

18. Amin N, van Duijn CM, Aulchenko YS (2007) A Genomic Background Based Method for Association Analysis in Related Individuals. PLoS One 2: e1274.

19. Aulchenko YS, de Koning D-J, Haley C (2007) Genomewide Rapid Association Using Mixed Model and Regression: A Fast and Simple Method For Genomewide Pedigree-Based Quantitative Trait Loci Association Analysis. Genetics 177: 577-585.

20. Willer CJ, Li Y, Abecasis GR (2010) METAL: fast and efficient meta-analysis of genomewide association scans. Bioinformatics 26: 2190-2191.

21. Higgins JP, Thompson SG, Deeks JJ, Altman DG (2003) Measuring inconsistency in meta-analyses. BMJ: British Medical Journal 327: 557.

22. Pruim RJ, Welch RP, Sanna S, Teslovich TM, Chines PS, et al. (2010) LocusZoom: regional visualization of genome-wide association scan results. Bioinformatics 26: 2336-2337.

23. (2012) An integrated map of genetic variation from 1,092 human genomes. Nature 491: 56-65.

24. Johnson AD, Handsaker RE, Pulit SL, Nizzari MM, O'Donnell CJ, et al. (2008) SNAP: a web-based tool for identification and annotation of proxy SNPs using HapMap. Bioinformatics 24: 2938-2939.

25. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, et al. (2007) PLINK: a tool set for whole-genome association and population-based linkage analyses. The American Journal of Human Genetics 81: 559-575.

26. Zhang W, Dai M, Fridberger A, Hassan A, DeGagne J, et al. (2012) Perivascular-resident macrophage-like melanocytes in the inner ear are essential for the integrity of the intrastrial fluid–blood barrier. Proceedings of the National Academy of Sciences 109: 10388-10393.

27. Shi X (2010) Resident macrophages in the cochlear blood-labyrinth barrier and their renewal via migration of bone-marrow-derived cells. Cell and Tissue Research 342: 21-30.

28. Degerman E, Rauch U, Göransson O, Lindberg S, Hultgårdh A, et al. (2011) Identification of new signaling components in the sensory epithelium of human saccule. Frontiers in neurology 2: 48.

29. Katoh Y, Takemori H, Horike N, Doi J, Muraoka M, et al. (2004) Salt-inducible kinase (SIK) isoforms: their involvement in steroidogenesis and adipogenesis. Molecular and Cellular Endocrinology 217: 109-112.

30. Berdeaux R, Goebel N, Banaszynski L, Takemori H, Wandless T, et al. (2007) SIK1 is a class II HDAC kinase that promotes survival of skeletal myocytes. Nat Med 13: 597-603.

31. Sasagawa S, Takemori H, Uebi T, Ikegami D, Hiramatsu K, et al. (2012) SIK3 is essential for chondrocyte hypertrophy during skeletal development in mice. Development 139: 1153-1163.

32. Walkinshaw DR, Weist R, Kim G-W, You L, Xiao L, et al. (2013) The tumor suppressor kinase LKB1 activates SIK2 and SIK3 to stimulate nuclear export of class IIa histone deacetylases. Journal of Biological Chemistry.

33. Clark K, MacKenzie KF, Petkevicius K, Kristariyanto Y, Zhang J, et al. (2012) Phosphorylation of CRTC3 by the salt-inducible kinases controls the interconversion of classically activated and regulatory macrophages. Proceedings of the National Academy of Sciences 109: 16986-16991.

34. Uebi T, Itoh Y, Hatano O, Kumagai A, Sanosaka M, et al. (2012) Involvement of SIK3 in Glucose and Lipid Homeostasis in Mice. PLoS One 7: e37803.

35. Cavalli-Sforza LL (2005) The Human Genome Diversity Project: past, present and future. Nat Rev Genet 6: 333-340.

Chapter 7: Epigenetics in age-related hearing impairment

Abstract

Epigenetic regulation of gene expression has been shown to change over time and has been associated with environmental exposures [1] and multiple complex traits [2,3]. Epigenome-wide association studies and studies of identical twins showing phenotypic discordance are the major study designs used to identify differentially methylated regions (DMRs) linked to the trait under study. Monozygotic (MZ) twin pairs are well matched for genetic variation and environmental exposure [4]. Loss of hearing ability with higher age is a complex disorder known to be heritable [5], and epigenetic regulation could explain the differences in hearing ability between identical twins as well as [4,6] in age of onset and magnitude of hearing ability seen in the elderly population [7,8].

Pure-tone audiograms were collected from female twins (n=1303, age range: 40-86 years) of the TwinsUK cohort. DNA methylation data based on the Illumina HumanMethylation27K array were available in 115 subjects (age range: 47-83 years). We performed an epigenome-wide association scan (EWAS) with hearing loss, using the first two principal components of the audiogram (PC1, PC2). We found that these two hearing traits were strongly associated with DNA methylation levels in the promoter regions of genes *TCF25* (cg01161216, $p=6.6x10^{-6}$), *FGFR1* (cg15791248, $p=5.7x10^{-5}$), *POLE* (cg18877514, $p=6.3x10^{-5}$) and *ACADM* (cg05467918, $p=1.72x10^{-6}$). Replication of these results in a second independent sample from TwinsUK (n=203, age range: 41-86 years), confirmed the presence of differentially methylated probes at *TCF25* ($p(replication)=8.5x10^{-5}$) and *POLE* (p(replication)=0.017). The association with *TCF25* was validated using methylation dependent immunoprecipitation and high throughput sequencing (MeDIPseq, p=0.04).

In a discordant monozygotic twin study, difference in hearing ability was correlated with DNA methylation differences within twin pairs (n=21 pairs). Differential methylation at *ACP6* (cg01377755, r=-0.75, $p=1.2x10^{-4}$) and *MEF2D* (cg08156349, r=-0.75, $p=1.4x10^{-4}$) showed the strongest correlation with differences in PC1, while PC2 discordance correlated strongly with differential methylation at *CCNDBP1* (cg12113132, r=-0.792, $p=2.5x10^{-5}$). Gene expression regulation of these genes by differential methylation could explain the late onset of age-related hearing impairment and the great variance in hearing ability.

In conclusion, we have identified differential methylation changes at several genes, which may shed light on epigenetic mechanisms operating in hearing ability with age.

Introduction

Epigenetics: history, mechanisms and definition

Epigenetics stems from the Greek word epigenetic, which translates as “on, above or around the gene” [9] and was first coined by Conrad Waddington, who defined epigenetics as a summary of “*events which lead to the unfolding of the genetic program for development*” [10]. For Waddington epigenetics explained how pluripotent cells could differentiate into unique cell-types despite having inherited identical DNA sequences. According to the ENCODE project [11] human DNA encodes roughly 20,700 protein coding genes, however, only a minority of these genes are expressed in a specific cell type at a given point in time. While each cell in an organism should have inherited an identical DNA sequence, expression profiles across cell types differ tremendously. More modern definitions of the term epigenetics include all molecular processes affecting gene expression without altering the nucleotide sequence of DNA [12,13]. Epigenetic modifications help to orchestrate the expression of genes required for the cell under given circumstances [14] primarily by altering chromatin structure. In contrast to the static DNA sequence, epigenetic modifications are relatively flexible and have been shown to adapt to environmental changes. Despite their ability to change, epigenetic modifications can be stably maintained over long time frames [15]. Several epigenetic mechanisms are recognised, including DNA methylation, histone modification, regulatory RNA, specific transcription factors and prions [16]. This chapter will focus primarily on the epigenetic change DNA cytosine methylation, one of the most widely studied epigenetic mechanisms due its high abundance and relative ease of measurement.

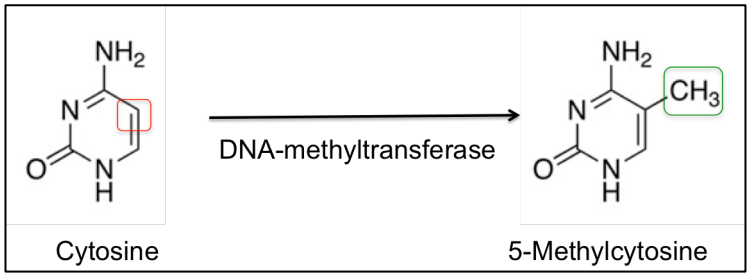


Figure 41 Cytosine methylation

Cytosine methylation involves the addition of a methyl-group (CH_3, labelled in green) by a DNA-methyltransferase to the 5th carbon atom (position labelled in red) of cytosine, forming 5-methylcytosine.

DNA methylation refers to the stable addition of a methyl-group (CH_3) to the 5th carbon atom of cytosine, resulting in 5-methylcytosine (Figure 41). The methylation of cytosines occurs primarily at CpG dinucleotides, which show an uneven distribution across the genome and often cluster in the promoter region of genes, where they are referred to as CpG islands (CpGI). CpGI are defined as sequences of ≥ 200 bps with a CG content of more than 50% [17]. Whereas 70-80% of CpG dinucleotides in the genome are methylated [18], CpGI in the promoter region of genes are mostly unmethylated . Methylation of CpGI promoters results in an unfavourable chromatin structure for the transcription machinery and transcription factors to access, therefore causing repression of gene expression. Furthermore, DNA methylation recruits methyl-CpG binding proteins, which in return can recruit secondary repressor proteins like histone de-acetylases [19]. Whereas hypermethylation of cancer repressor genes has been detected in several forms of cancers [20], hypomethylation of generally methylated gene promoters can lead to over-expression of genes [21]. In addition, methylation of repetitive sequences and retrotransposons supports DNA stability by silencing these parts of the genome, thus preventing DNA rearrangements [21,22,23]. More importantly, DNA methylation is an essential process during development of an organism, allowing cells to differentiate and adapt their gene expression to the requirements of their particular tissue [14,24]. This process is complex however and outside promoter regions methylation often leads to over-expression.

Cytosine methylation is mediated by three DNA methylases (DNMTs): DNMT1, DNMT3a and DNMT3b. DNMT1 shows a high affinity to hemi-methylated DNA strands [25,26]. It co-localises with proliferating cell nuclear antigen during DNA replication and copies the DNA methylation profile of the mother to the newly synthesized daughter strand, thus being referred to as maintenance DNMT [26]. Members of the DNMT3 family of methylases show equal affinities for hemi- and unmethylated DNA sequences

[27,28,29,30]. They are therefore referred to as de-novo-methylases and play an essential role in establishment of DNA methylation after methylation erasure in the blastocyst and silencing of transposable elements [27,30]. Whereas the process of DNA methylation is relatively well explored, the process of epigenetic reprogramming (i.e. erasure and re-establishment of parental epigenetic marks in the blastocyst) is still poorly understood [31].

While DNMTs are responsible for methylation transmission during mitosis, trans-generational epigenetic transmission is more complicated [32]. The most prominent example of transgenerational epigenetic transmission involves the agouti viable yellow (A^{vy}) allele in mice. This allele developed from a retrotransposon insertion upstream of the agouti gene, which regulates coat colour and if overexpressed results in obesity. The agouti promoter is located in the retrotransposon insertion, resulting in the agouti gene expression being regulated by the methylation status of the promoter sequence [33]. It was shown that female mice with a high agouti expression (causing yellow coat colour) produced a higher percentage of yellow offspring than dams with lower expression. This indicates incomplete erasure of epigenetic marks at the A^{vy} allele in the female germline and thus epigenetic inheritance at this locus. Maternal effects post fertilization could not explain this effect [33].

A similar study in sheep showed that periconceptional dietary restriction of methionine, vitamin B_{12} and folate led to wide-spread epigenetic modifications in the adult offspring [34]. The three restricted nutrients are included in the methionine cycle producing the primary methyl group donor, S-adenosylmethionine, in DNA-methylation [35]. The epigenetic modifications seen in adult offspring sheep were further associated with increased body weight, insulin-resistance, high blood-pressure and differences in immune response. Interestingly, phenotypic differences were more prominent in male than in female adult offsprings [34].

Similar results have been observed in humans conceived during the Dutch winter famine (1944-1945). Six decades after their periconceptional exposure to famine in uterus, exposed individuals showed significant hypomethylation ($p \leq 1.5 \times 10^{-4}$) at the imprinted *IGF2* gene in comparison to their unexposed same sex siblings [36]. Epigenetic heritability studies in humans have shown an increased epigenetic correlation within dichorionic MZ twin pairs, who split early in uterus, compared to DZ pairs across 6000 genes extracted from buccal epithelial cells [37]. However, DNA methylation profiles measured longitudinally on a gene specific level showed less to no

indication of increased epigenetic correlation in MZ compared to DZ twin pairs [1]. So far unlike animal models, the human studies lack convincing evidence of definite epigenetic trans-generational evidence.

Use of twins in epigenetic studies

One major limitation of epigenetic studies is the lack of a reference sequence. Due to the flexibility of epigenetic modifications with environment, age, disease and various other factors, it is impossible to define which modifications form the standard for an individual. While animal studies can control for environmental factors under laboratory conditions, this is unethical to apply to humans. In addition, the influence of age, gender and genotype variation will bias epigenetic analyses [38,39]. Therefore, three main study designs have been preferred for epigenetic analyses: family-studies, longitudinal studies of the same individuals or MZ twin studies. MZ twin siblings share their genetic material and a high proportion of environmental exposures, like time in uterus, familial environment and early nutrition. In addition, they are perfectly matched for age and gender. Particularly in the case of MZ twins discordant for a specific phenotype, epigenetic modification could explain why one twin sibling develops a trait while the other one does not, despite identical genetic material. Furthermore, MZ and DZ twin pairs can give a measure of heritability of epigenetic marks. Studies on various phenotypes have proven the usefulness of MZ twins in this regard [1,4,6,40]. In a study of 40 MZ twin pairs of Spanish ancestry (age range: 3-74) it could be shown that at a young age, epigenetic profiles were very similar within twin pairs but grew more discordant with increasing age [6]. Furthermore, although numbers were small twin pairs with discordant lifestyles showed greater differences in epigenetic marks than twin siblings with similar lifestyles [6].

Methods to measure DNA methylation

To determine the methylation status of a specific CpG site or CpG dinucleotides across the genome reliable methods are required. Three major DNA methylation assays are available: restriction digest with methylation sensitive restriction enzymes, affinity enrichment by methyl-group specific antibodies or proteins and bisulfite conversion [41].

Bisulfite conversion is based on the observation that sodium bisulfite treatment of DNA converts unmethylated cytosine to uracil and spares methylated cytosines. Thus a change in DNA is introduced dependent on DNA methylation status at the respective cytosine. The converted DNA will be amplified in a polymerase chain reaction and subsequently sequenced. It is critical that all unmethylated cytosines are converted to

uracil to avoid false negatives. High throughput methods developed for bisulfite treated DNA allow simultaneous screening of DNA methylation at up to 480.000 CpG sites. In this chapter, DNA methylation was measured using two assays based on bisulfite conversion, the Illumina Infinium HumanMethylation27K (27K) and HumanMethylation450K (450K) Bead Chips. This work was performed as part of the EpiTwin project (http://www.epitwin.eu/index.html). DNA methylation was measured from venous blood samples collected from TwinsUK volunteers. Both assays have been applied previously to measure DNA methylation epigenome-wide [40,42]. The Illumina Infinium 27K array measures methylation at 27.758 CpG sites, covering 14475 consensus coding sequences and cancer genes. This array primarily covers CpG sites located in promoter regions of genes with on average two CpG sites per consensus coding sequence and three to twenty assays per cancer gene [43]. The Illumina Infinium 450K array, in contrast, covers 485577 methylation sites in 99% of RefSeq genes (21231 genes) with an average of 17.2 CpG sites per gene region. In addition, its successor the 450K array covers not only CpG sites in the promoter regions of genes, but also CpG sites in the first exon, gene body and 5' and 3' untranslated regions [44]. Both assays differ further in the probe design used. While the Illumina 27K Bead chip uses exclusively Illumina I beadtypes, with one bead per methylated and unmethylated CG site measured [43,45], the 450K chip applies both Illumina I and Illumina II beadtypes. Illumina II beadtypes use only one probe per CpG site with differential colour signals indicating the methylation status of the annealing sequence [44] (Figure 42).

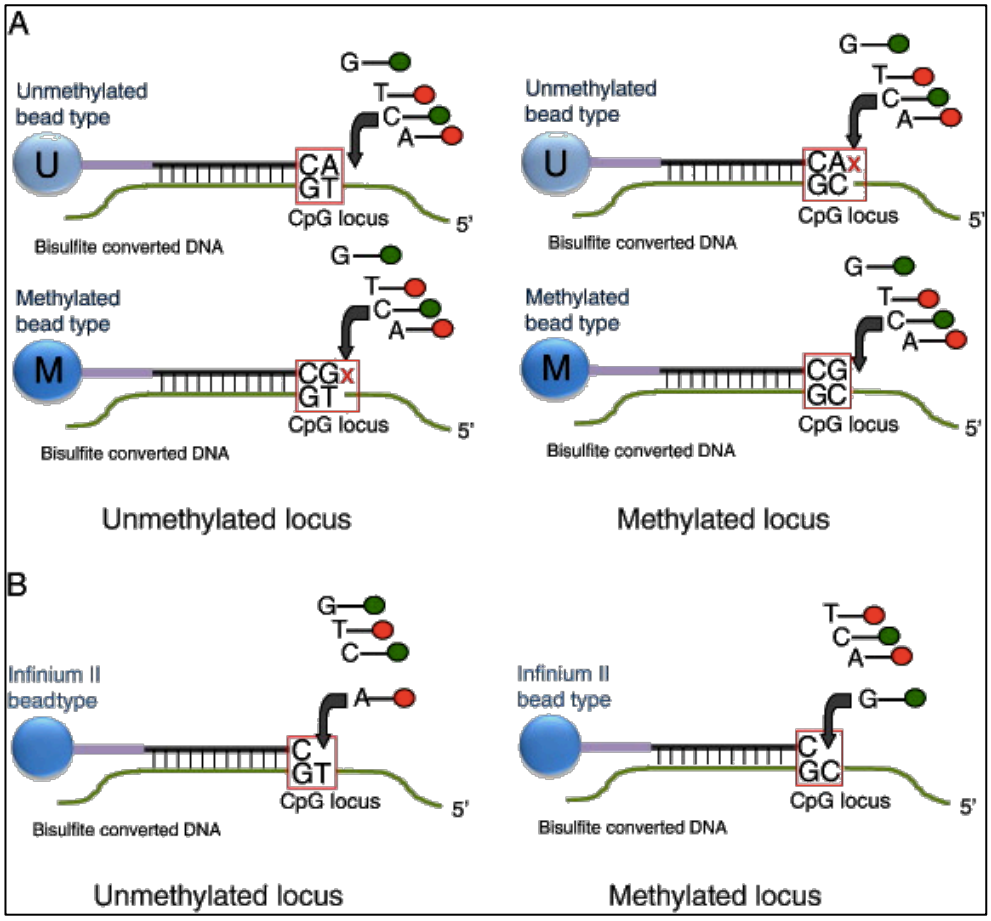


Figure 42 Illumina Infinium I and II methylation assay probe designs

A. The Illumina Infinium I beadtype methylation assay uses 2 probes per CpG site to detect methylation, while in **B.** Illumina Infinium II beadtype assay applies one probe per CpG site using different colour signals to differentiate between methylated and unmethylated loci (graphic adapted from M. Bibikova [44]).

Methylated DNA Immunoprecipitation (MeDIP) is an affinity enrichment method that uses antibodies to bind to methylated DNA sequences [46]. To prepare samples for this affinity treatment, extracted DNA has to be sheered into short fragments (300-1000 bp in length) by sonication and denatured to produce single-stranded DNA fragments. These single stranded DNA fragments are subsequently treated with the respective antibody (i.e. 5-methylcytidine antibody) and antibody bound fragments enriched using immunoprecipitation techniques [46]. The resulting enriched methylated DNA sequences can then be identified by the use of microarrays or next generation sequencing techniques (MeDIPseq) [47].

Epigenetics of hearing disorders

ARHI is a common complex disorder, which has been intensely studied using genetic [48,49,50,51,52] and environmental tools [53,54]. The search for genetic risk loci of ARHI was of limited success despite relatively large cohorts and promising study designs. In addition, the few identified risk loci fail to explain the large differences in

severity of HL seen in many ageing populations. While studies in mice and humans show that genotype can determine sensitivity to environmental factors like noise [55] and ototoxic medication [56,57], epigenetic modifications could further explain changes in gene expression upon environmental factors like smoking behaviour. Furthermore, ARHI develops in individuals with previously normal hearing at various times of onset and intensity. Epigenetic modifications could accumulate over a lifetime in association with environmental exposure. Epigenetic changes might also explain how hearing in genetically identical individuals, like monozygotic twin siblings, diverges with increasing age independent of environmental exposure. A Previous study of one MZ twin pair discordant for Alzheimer's disease showed reduced DNA methylation levels in neuronal nuclei in the neocortex of the diseased twin compared to his unaffected sibling [58]. Similar findings have been reported for Schizophrenia discordant and concordant MZ twin pairs. In this study DNA methylation at the 5'-regulatory region of the Dopamine D2 receptor gene was investigated in two MZ twin pairs, one concordant and the other pair discordant for schizophrenia [59]. DNA methylation patterns in the discordant affected twin were more similar to the patterns observed in the concordant affect siblings than to the DNA methylation seen in its own sibling [59]. Particularly in hearing ability with age the moderate heritability estimates have yet to be explained by genetic associations with the trait [60]. Epigenetic modifications might explain for part of the missing heritability currently attributed to genetic variation [60]. In conclusion, several factors favour an epigenetic mechanism involved in hearing loss with age.

A role of epigenetic modifications in ARHI on a genome-wide level has not been formally documented, however, reviews of epigenetics in hearing phenotypes have suggested it [7,8]. Besides, several forms of syndromic hearing impairment have been linked to epigenetic processes [7]. In Stickler syndrome type 1, a disorder involving hearing loss to various extends, an exceptionally high rate of cytosine to threonine point mutations were detected, which lead to stop codons in the responsible gene. It could be shown that these base transitions resulted from mutations of methylated CGA codons by cytosine deamination [61]. In Rett syndrome, a disorder characterised by specific abnormal hand movements, motor and neurological defects, mutations in the methyl-CpG binding protein (MeCP2) have been determined [62]. MeCP2 interacts with DNMT1 and histone-deacetylase and can bind methylated CpG dinucleotides to repress transcription. In addition, many mutations resulting in Rett syndrome occur at methylated CpG sites, highlighting the fact that methylated CpGs are prone to mutations [8]. Further epigenetic mechanisms involved in hearing phenotypes have been summarised in more detail elsewhere [7,8].

The aim of this chapter was to explore the role of DNA methylation in ARHI. Two study designs were used: an epigenome-wide association study (EWAS) associating hearing ability with DNA methylation genome-wide and an MZ twin pair discordance analysis, to identify differentially methylated CpG sites within MZ twin pairs in correlation with intra-pair hearing ability.

Methods

Subjects and hearing phenotypes

Participants were recruited from females of the TwinsUK cohort. Hearing data were collected in the form of pure-tone audiograms, assessing pure-tone thresholds at frequencies 0.125-8 kHz. Log-transformed pure-tone thresholds were summarised in a principal component analysis, with PC1 and PC2 representing the overall threshold shift and slope of the audiogram, respectively.

DNA methylation profiles

Measurement of DNA methylation in TwinsUK samples was performed as part of the EpiTwin project (http://www.epitwin.eu/index.html) under the lead of Prof. Tim Spector and Dr. Jordana Bell. The 450K data was assayed at the Wellcome Trust Sanger Institute Cambridge under the lead of Prof. Panos Deloukas. The DNA methylation data processing as presented under the DNA methylation profiles section was kindly provided for this project.

DNA methylation from venous whole blood samples was measured using two different DNA methylation assays, the 27K BeadChip (26,690 CpG sites) and the Infinium 450K BeadChip kit. For both assays, the DNA methylation level at a specific CpG site is expressed as β value, representing the ratio of methylated probe signal (S_M) over the sum of methylated and unmethylated probe signal (S_U) (Equation 1). β scores ranged from 0 to 1, with 0 indicating no methylation and 1 representing a fully methylated probe.

$$\beta = \frac{S_M}{S_U + S_M}$$ [Equation 1. Methylation β]

To identify potential confounders of the Illumina Infinium HumanMethykation27K array, a principal component analysis (PCA) was performed using the normalised DNA methylation values. The first five principal components resulting from this analysis were examined for association with confounders by correlation analysis with following covariates: chronological age, methylation chip and position of sample on the chip. Both methylation chip and position of sample on the chip were found to be significantly

correlated with the first two principal components from this analysis and so these covariates were included as fixed effects in further analyses [40].

The same analysis was performed for the Illumina Infinium 450K array with covariates age, chip, position of sample on the chip and bisulfite conversion levels (unpublished data). Chip, position on the chip and bisulfite conversion levels were significantly associated with the first 3 principal components and were included as fixed effects in the linear mixed effects model for future analysis of this dataset.

Epigenome-wide association study

DNA methylation was measured for 26690 DNA methylation probes from the HumanMethylation27K chip, each of which mapped to a different CpG dinucleotide in the human genome (hg18)[63]. After exclusion of probes mapping to the X-chromosome and probes with missing data performed by Dr. Jordana Bell and Pei-Chien Tsai, 24641 autosomal probes remained for the EWAS [40]. To determine associations between hearing ability and DNA methylation a linear mixed effect model was applied, where DNA methylation levels at individual CpG-sites were examined as a function of hearing ability (PC1 or PC2, respectively), with chip, position on the chip and chronological age included as fixed effects, and family identifier and zygosity as random effects. DNA methylation was transformed to standard normal per probe using a quantile normalisation. To exclude associations with DNA methylation due to covariates other than hearing, the full model was compared to a null model, in which hearing (PC1 or PC2, respectively) was excluded as a predictor variable. The null and the full model were compared for model fit in an analysis of variance (ANOVA). Only associations where the full model fitted the data significantly better ($p(ANOVA) \leq 0.05$) than the null model were reported. For each significantly associated probe, the effect size (beta), standard error of the effect (se) and the p-value from the ANOVA comparing full and null model were reported (Table 40 and Table 41, 27K).

To adjust for multiple testing in the discovery EWAS initially a Bonferroni corrected significance threshold assuming 24,641 independent tests ($p=0.05/24641=2.03 \times 10^{-6}$) was assumed epigenome-wide significant. However, the 27K array contains on average 2 probes per promoter and high levels of co-methylation between neighbouring probes have been reported recently [63]. It was therefore decided to consider two further Bonferroni corrected thresholds: a genome-wide significant threshold correcting for 14,495 independent genes ($p=0.05/14495=3.45 \times 10^{-6}$) and a

genome-wide suggestive threshold correcting for 14,495 independent genes ($p=0.1/14495=6.90x10^{-6}$).

To confirm that the associated probes were not age-dependent differentially methylated regions (age DMRs), we determined the significance of model fit (p(no age)) by comparing the full and null model in an ANOVA after excluding age as a fixed effect in both models (Table 40 and Table 41, p-value (no age)). In addition, the ten most strongly associated probes in both PC1 and PC2 EWAS were checked against previously reported age DMRs in these data [40].

To exclude an underlying association between genetic variation and hearing at differentially methylated regions, corresponding loci were checked for association in genome-wide association scans with PC1 and PC2 data, respectively (chapter 5).

Replication study

The top 10 associated probes from the discovery EWAS for PC1 and PC2 were taken forward for replication. To determine associations between hearing ability and DNA methylation a linear mixed effect model was applied. In the full linear mixed effect model, the DNA methylation β at the 10 selected probes was expressed as a function of hearing (PC1;PC2), incorporating fixed (chronological age, chip-number and position on chip) and random (family identifier and zygosity) effects. DNA methylation was transformed to standard normal per probe using a quantile normalisation. To exclude associations with DNA methylation due to covariates other than hearing, we compared the full model to a null model, in which hearing was excluded as a predictor variable. The null and the full model were compared for model fit in an ANOVA. For each of the 10 probes, the effect size (beta), standard error of effect (se) and the p-value from the ANOVA comparing full and null models were reported (Table 40 and Table 41, 450K). Replication of association was assumed if the direction of effect was concordant in the discovery and replication sample and if association signals in the replication cohort passed nominal significance ($p \leq 0.05$).

To confirm that the replicating associated probes were not age DMRs, the significance of model fit (p(no age)) after exclusion of age as a fixed effect in both full and null model was determined by ANOVA for replicating probes (Table 40).

To determine the joint effect of discovery and replication study for the ten most strongly associated probes, a meta-analysis was conducted in the program METAL [64] based on the reverse variance option (Table 40 and Table 41, meta-analysis) The reported meta-analysis results include the direction of effect for both discovery and replication study, the joint beta with corresponding standard error and significance of joint association.

Whole blood cell subtype heterogeneity

Previous studies have reported that association with DNA methylation measured in whole blood samples can be driven by blood cell subtype heterogeneity [65]. To adjust for this possible bias, eosinophil, lymphocyte, neutrophil and monocyte concentrations in blood samples were included as fixed effects in the full and null models for the ten most highly associated probes. Full blood cell subtype counts were available in 106 individuals from the discovery EWAS sample. This test aimed to determine whether blood cell heterogeneity alone could explain the differences in DNA methylation.

DMR validation using methylated DNA immunoprecipitation sequencing (MeDIPseq)

To further validate the findings from the EWAS (27K) and replication study (450K) using an alternative technique, the top ranked association with DNA methylation at *TCF25* was also explored using MeDIPseq [47]. The MeDIPseq validation sample consisted of 46 unrelated healthy females with PTA scores and previously published MeDIPseq profiles [66]. None of these subjects had been included in either discovery or replication samples. MeDIPseq DNA methylation levels were generated and quantified as previously described [66]. This data was kindly provided from the EpiTwin project in collaboration with the Beijing Genomics Institute who performed the genotyping. Relative methylation scores in a 1kb region on chr 16 (chr16: 88466501-88467500 on hg 18) overlapping probe cg01161216 (chr16: 88466949 on hg 18) were explored for association with PTA. A linear fixed effect model was applied, where the DNA methylation signal at the locus surrounding the chromosomal position of probe cg01161216 was regressed on hearing ability (PC1), adjusted for age. To exclude an association of DNA methylation with other factors than hearing, the full model was compared to a null model, excluding hearing as a predictor variable. The null and full models were compared for model fit using ANOVA.

Effect of DNA methylation on gene expression

To investigate the influence of DNA methylation on gene expression at selected genes, we tested the correlation between DNA methylation probes and gene expression in

skin tissue collected in TwinsUK as part of the Multiple Tissue Human Expression Resource (MuTHER) (http://www.muther.ac.uk) [67].

Quantile normalised gene expression in skin was adjusted for experimental batch effect and RNA concentration in the tissue sample and residuals examined for an association with DNA methylation residuals (adjusted for chip and position on the chip) at the corresponding probes using Pearson correlation. Furthermore, we tested for an association between skin expression residuals and PC1 values using correlation analysis, to test for an effect of gene expression on phenotype.

Exploring methylation changes in monozygotic twins

All MZ twin pairs from the discovery sample were selected for the MZ discordance analysis. Within twin pair DNA methylation difference per probe was calculated as the difference in DNA methylation residuals (adjusted for chip and position on the chip) between twin sister 1 and twin sister 2. DNA methylation residuals were calculated from quantile normalised β values per probe. The association between differences in DNA methylation and differences in PC1 or PC2 was examined using Spearman correlation (Table 42).

Results

Subjects

As described in Chapter 2, pure-tone audiograms were collected for 1303 females from the TwinsUK cohort. Of these, 115 individuals had 27K data available for epigenetic analysis. Volunteers had a mean age of 63.57 years (±7.05 years of standard deviation from the mean, age range 47-83 years). The study sample included 25 dizygotic twin (DZ) pairs, 21 MZ twin pairs as well as 23 unpaired twins.

For the replication cohort, data from 203 females having pure-tone audiometry and Illumina Infinium Humanmethylation450K data, which were not included in the discovery EWAS (27K), was used. This sample included 61 MZ twin pairs, 22 DZ twin pairs and 37 unpaired twins. Age of subjects at hearing test ranged from 41 to 86 years with a mean age of 63.21years ±8.87 years standard deviation (SD).

The validation sample (MeDIPseq) consisted of 46 unrelated females from TwinsUK with an age range of 43 to 86 years (mean age: 62.28 ±7.86 SD). Population

characteristics of the discovery (27K), replication (450K) and validation (MeDIPseq) samples are summarised in Table 39.

Table 39 Population characteristics for the discovery, replication and validation samples

sample	zygosity	n	age at DNA extraction		age at hearing test		PC1 ±SD	PC2 ±SD
			mean ±SD	range	mean ±SD	range		
discovery (27K)	MZ	42	55.43 ±6.93	45-68	62.00 ±6.60	50-72	-0.28 ±1.47	-0.18 ±1.27
	DZ	50	57.68 ±8.88	33-80	64.32 ±7.68	47-83	0.72 ±1.53	-0.17 ±1.58
	unpaired	23	56.91 ±7.35	43-70	64.83 ±6.12	50-75	0.29 ±1.78	-0.30 ±1.20
	Total	**115**	**56.70 ±7.91**	**33-80**	**63.57 ±7.05**	**47-83**	**0.27 ±1.61**	**-0.20 ±1.39**
replication (450K)	MZ	122	55.64 ±8.83	37-73	63.82 ±8.79	46-82	0.60 ±2.13	-0.21 ±1.46
	DZ	44	52.88 ±10.59	33-78	60.86 ±10.58	41-86	0.21 ±2.48	-0.29 ±1.36
	unpaired	37	55.87 ±6.20	42-66	63.97 ±6.28	49-75	0.21 ±1.74	-0.41 ±1.05
	Total	**203**	**55.09 ±8.87**	**33-78**	**63.21 ±8.87**	**41-86**	**0.45 ±2.15**	**-0.27 ±1.37**
validation (MeDIPseq)	**unpaired**	**46**	**60.02 ±7.85**	**41-83**	**62.28 ±7.86**	**43-86**	**-0.10 ±2.08**	**-**

Demographic characteristics of the discovery (27K DNA methylation bead chip), replication (450K DNA methylation bead chip) and validation (methylated DNA immunoprecipitation and high throughput sequencing (MeDIPseq) samples are shown. Sample zygosity is shown (monozygotic (MZ) and dizygotic (DZ) twins) as well as unpaired twins. Mean principal component 1 (PC1) values and standard deviation (SD) from the mean, representing the overall threshold shift in the audiogram, are given.

DNA methylation profile

Genome-wide DNA methylation (Illumina Infinium Human Methylation 27K) levels were measured in the discovery sample. Most CpG sites investigated were unmethylated (β<0.3, 68.9% of probes), while a minority of probes were fully methylated (β>0.7, 19.9% of probes) or hemi-methylated (β: 0.3-0.7, 11.2% of probes).

Epigenome-wide association study (EWAS)

Epigenome-wide association was assessed in a regression analysis comparing genome-wide DNA methylation levels (β) with hearing PC1 and PC2 separately. DNA methylation at 2519 and 1181 probes was nominally associated (p(ANOVA)≤0.05) with hearing ability as measured by PC1 and PC2, respectively. A Manhattan Plot of the EWAS with hearing PC1 and PC2 can be seen in Figure 43 and Figure 44, respectively. The ten most highly associated differentially methylated probes are located above the blue horizontal line corresponding to a p-value of $p=6.98x10^{-5}$ for the PC1 EWAS and a significance level of $p=2.55x10^{-4}$ for the PC2 EWAS. The red line marks a significance threshold of $p=10^{-5}$ for both Manhattan plots and CpG sites with significant associations passing this threshold depicted as red dots (Figure 43 and Figure 44).

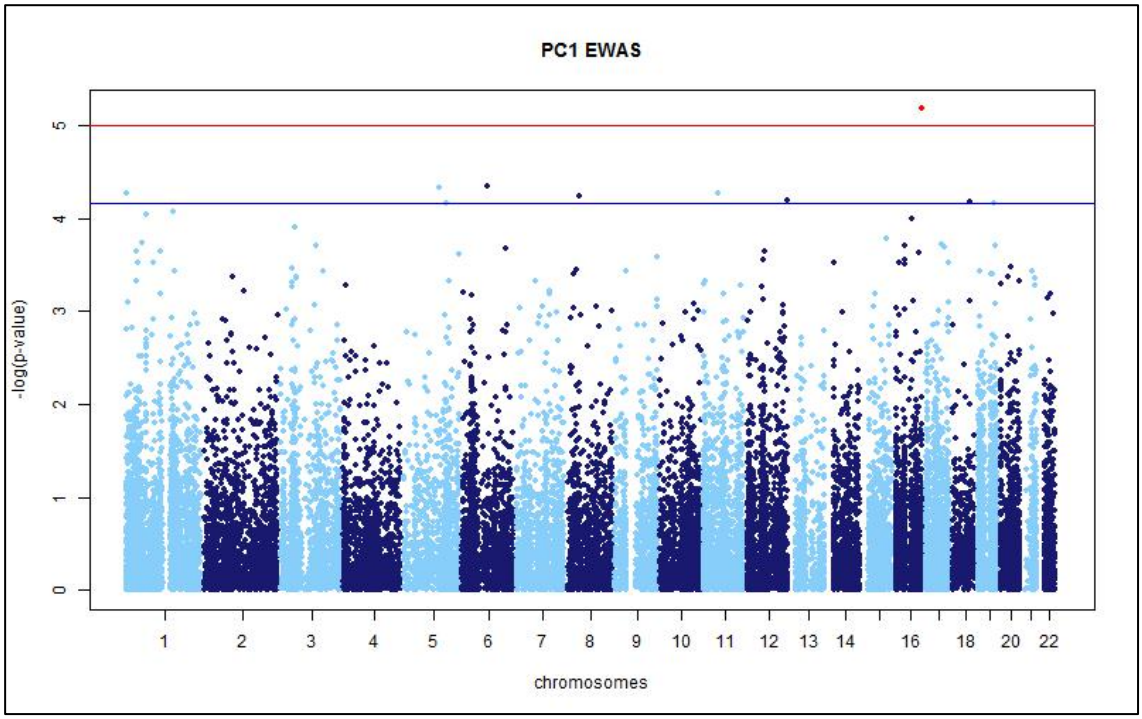


Figure 43 Manhattan plot of PC1 EWAS (27K) results

The Manhattan plot for epigenome-wide association with PC1 depicts the significance of association as the negative logarithm of the p-value versus chromosomal location for each of the 24641 tested DNA-methylation probes. The red line defines a significance threshold of $p=10^{-5}$. The ten most highly associated probes are located above the blue line corresponding to a p-value of $p=6.98x10^{-5}$.

PC1 was most significantly associated with DNA methylation at probe cg01161216, located in the promoter region of the transcription factor 25 (*TCF25*) gene (beta±se= -0.245±0.052, $p=6.60x10^{-6}$). Further associations between differential DNA methylation and hearing threshold shift were observed for CpG sites in the phosphoglucomutase 3 (*PGM3*) gene (beta±se=-0.261±0.056, $p=4.46x10^{-5}$), the cysteine dioxygenase type 1 (*CDO1*) gene (beta±se=-0.238±0.056, $p=4.67x10^{-5}$), the nucleolar complex associated 2 homolog (*NOC2L*) gene (beta±se=-0.200±0.048, $p=5.38x10^{-5}$), the myosin binding protein C (*MYBPC3*) gene (beta±se=-0.190±0.045, $p=5.44x10^{-5}$), the fibroblast growth factor receptor 1 (*FGFR1*) gene (beta±se=-0.242±0.058, $p=5.73x10^{-5}$), the DNA polymerase epsilon catalytic subunit (*POLE*) gene (beta±se=-0.163±0.039, $p=6.33x10^{-5}$), vacuolar protein sorting 4 homolog B (*VPS4B*) gene (beta±se=0.196±0.048, $p=6.55x10^{-5}$), the heterogeneous nuclear ribonucleoprotein A0 (*HNRNPA0*) gene (beta±se=0.136±0.033, $p=6.90x10^{-5}$), and probe cg25017250 (beta±se=-0.232±0.055, $p=6.98x10^{-5}$) mapping to the apolipoprotein C-4 (*APOC4*) gene. The ten most highly associated PC1 EWAS probes are listed in Table 40. After exclusion of chronological age as a fixed effect, association of DNA methylation with PC1 remained significant for all of the ten most strongly associated probes (Table 40).

Table 40 Results for PC1 EWAS, EWAS replication and meta-analysis of epigenome-wide association for the ten most highly associated probes

probe	gene	27K (n=115)		450K (n=203)		meta-analysis (n=318)		
		beta ± SE	p-value p-value (no age)	beta ± SE	p-value p-value (no age)	dir	beta ± SE	p-value
cg01161216	*TCF25*	-0.245 ±0.052	6.60E-06 4.98E-04	-0.124 ±0.031	8.55E-05 1.06E-08	- -	-0.155 ±0.027	4.89E-09
cg25383093	*PGM3*	-0.261 ±0.056	4.46E-05 7.46E-05	-0.008 ±0.034	8.08E-01 7.66E-02	- -	-0.075 ±0.029	9.80E-03
cg07644368	*CDO1*	-0.238 ±0.056	4.67E-05 2.06E-03	0.023 ±0.039	5.50E-01 4.40E-01	- +	-0.061 ±0.032	5.80E-02
cg19923810	*NOC2L*	-0.200 ±0.048	5.38E-05 3.24E-05	-0.0253 ±0.031	4.26E-01 8.16E-01	- -	-0.077 ±0.026	3.05E-03
cg21370143	*MYBPC3*	-0.190 ±0.045	5.44E-05 1.03E-05	-0.052 ±0.034	1.30E-01 1.62E-01	- -	-0.102 ±0.027	1.95E-04
cg15791248	*FGFR1*	-0.242 ±0.058	5.73E-05 5.01E-05	-0.015 ±0.038	6.96E-01 5.13E-01	- -	-0.084 ±0.032	8.46E-03
cg18877514	*POLE*	-0.163 ±0.039	6.33E-05 9.08E-04	-0.068 ±0.028	1.70E-02 2.83E-02	- -	-0.101 ±0.023	1.20E-05
cg05934874	*VPS4B*	0.196 ±0.048	6.55E-05 8.98E-04	-0.088 ±0.030	3.67E-03 1.88E-02	+ -	-0.007 ±0.025	7.80E-01
cg12241297	*HNRNPA0*	0.136 ±0.033	6.90E-05 4.52E-04	-0.064 ±0.029	2.65E-02 3.18E-02	+ -	0.023 ±0.022	2.80E-01
cg25017250	*APOC4*	-0.232 ±0.055	6.98E-05 1.06E-03	-0.040 ±0.036	2.71E-01 1.71E-02	- -	-0.097 ±0.030	1.25E-03

This tables summarises the ten differentially methylated regions most highly associated with PC1 in the discovery EWAS (27K). In the discovery EWAS (27K) association of DNA methylation was associated with PC1 in a linear mixed effect model adjusted for batch effects, chronological age and subject relatedness. The ten most highly associated probes are characterised by the gene they localise to. The effect (beta), standard error of the effect (se) and significance of model fit (p-value) are reported. To confirm that association was not driven by association with chronological age, significance of model fit excluding age as a model parameter (p-value (no age)) was added. The ten most highly associated probes were taken forward for replication in an independent sample of 203 subjects (n=203) with 450K DNA methylation bead chip data available (450K). The effect (beta) standard error of the effect and significance of model fit (p-value) in the replication sample (450K) are reported. To confirm that association in the replication sample (450K) was not driven by association with chronological age, significance of model fit excluding age as a model parameter (p-value (no age)) was added The associations reported for the discovery EWAS (27K) and replication (450K) sample were meta-analysed (meta-analysis) using an inverse variance scheme. The direction of effect for both samples (dir), combined effect (beta), standard error of the combined effect (se) and significance of the combined association (p-value) are listed for the meta-analysis. Association of DNA methylation and hearing PC1 was replicated for probes cg01161216 and cg18877514 (highlighted in grey).

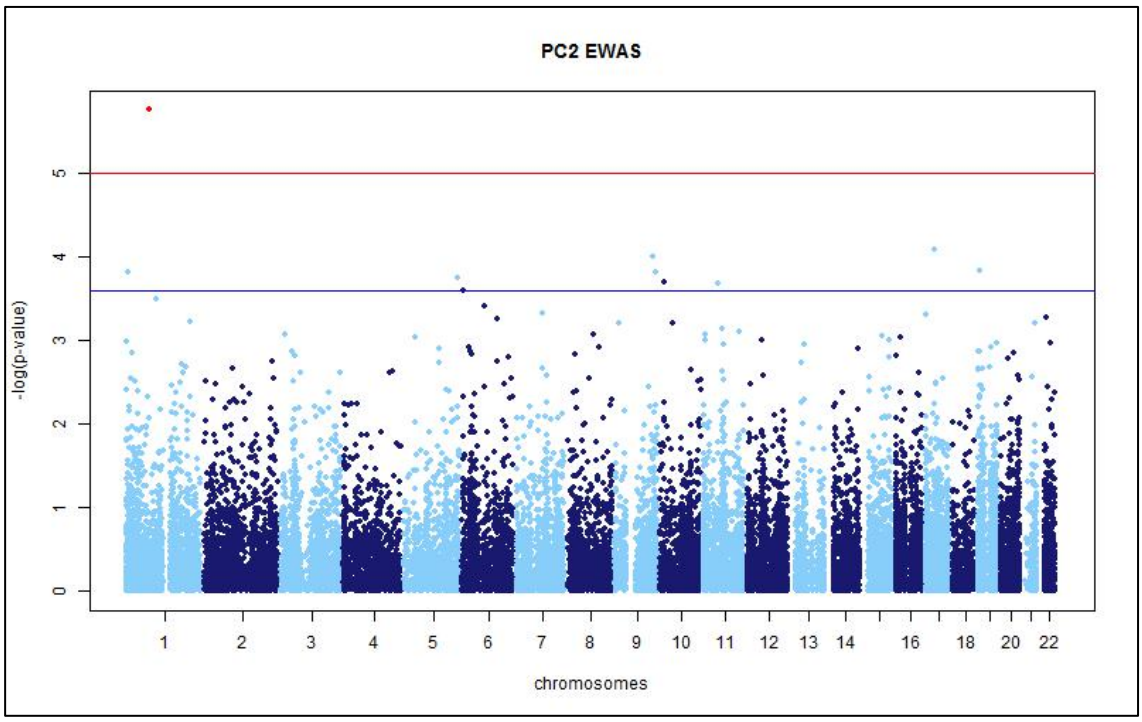


Figure 44 Manhattan plot of PC2 EWAS (27K) results

The Manhattan plot for epigenome-wide association with PC2 depicts the significance of association as the negative logarithm of the p-value versus chromosomal location for each of the 24641 tested DNA-methylation probes. The red line defines a significance threshold of $p=10^{-5}$. The ten most highly associated probes are located above the blue line corresponding to a p-value of $p=2.55x10^{-4}$.

PC2 was most significantly associated with differential DNA methylation at probe cg05467918 located in the acyl-CoA dehydrogenase (*ACADM*) gene (beta±se=0.323±0.063, $p=1.72x10^{-6}$). Additional associations between PC2 and differentially methylated CpG sites were observed for the peroxisome assembly protein 12 (*PEX12*) gene (beta±se=0.175±0.043, $p=8.04x10^{-5}$), the actin related protein 2/3 complex subunit 5-like (*ARPC5L*) gene (beta±se=0.191±0.047, $p=9.90x10^{-5}$), the CUGBP Elav-like family member 5 (*CELF5*) gene (beta±se=-0.274±0.070, $p=1.47x10^{-4}$), open reading frame 174 on chromosome 1 (*C1orf174*) (beta±se=0.171±0.044, $p=1.49x10^{-4}$), openreading frame 116 on chromosome 9 (*C9orf116*) (beta±se=0.185±0.047, $p=1.50x10^{-4}$), MAX dimerization protein 3 (*MXD3*) gene (beta±se=0.167±0.043, $p=1.79x10^{-4}$), the BEN domain containing 7 (*BEND7*) gene (beta±se=-0.208±0.053, $p=1.96x10^{-4}$), the DNA damage-binding protein 2 (*DDB2*) gene (beta±se=0.251±0.065, $p=2.05x10^{-4}$) and the PRP4 pre-mRNA processing factor 4 homolog B (*PRPF4B*) gene (beta±se=0.173±0.046, $p=2.55x10^{-4}$). The ten DMRs most highly associated with PC2 in the EWAS are listed in Table 41. After exclusion of chronological age as a fixed effect, association of DNA methylation with PC2 remained significant for all of the ten most highly associated probes (Table 41).

Table 41 Results for PC2 EWAS, EWAS replication and meta-analysis of epigenome wide association for the ten most highly associated probes

probe	gene	27K (n=115)		450K (n=203)		meta-analysis (n=318)		
		beta ± SE	p-value p-value (no age)	beta ± SE	p-value	dir	beta ± SE	p-value
cg05467918	*ACADM*	0.323 ±0.063	1.72E-06 5.47E-06	0.029 ±0.034	4.00E-01	++	0.095 ±0.030	1.45E-03
cg21116410	*PEX12*	0.175 ±0.043	8.04E-05 1.68E-03	-0.094 ±0.039	1.90E-02	+-	0.027 ±0.029	3.43E-01
cg03916787	*ARPC5L*	0.191 ±0.047	9.90E-05 8.23E-05	0.048 ±0.038	2.03E-01	++	0.105 ±0.030	4.04E-04
cg06734812	*CELF5*	-0.274 ±0.070	1.47E-04 5.59E-04	0.084 ±0.049	8.62E-02	-+	-0.034 ±0.040	4.01E-01
cg25762395	*C1orf174*	0.171 ±0.044	1.49E-04 4.42E-05	0.019 ±0.041	6.46E-01	++	0.090 ±0.030	2.80E-03
cg12438037	*C9orf116*	0.185 ±0.047	1.50E-04 1.03E-03	0.008 ±0.043	8.51E-01	++	0.089 ±0.032	5.20E-03
cg02693857	*MXD3*	0.167 ±0.043	1.79E-04 2.97E-04	0.067 ±0.042	1.16E-01	++	0.116 ±0.030	1.16E-04
cg14784653	*BEND7*	-0.208 ±0.053	1.96E-04 2.86E-04	-0.008 ±0.045	8.56E-01	--	-0.092 ±0.034	7.46E-03
cg19486271	*DDB2*	0.251 ±0.065	2.05E-04 1.18E-03	-0.093 ±0.055	9.62E-02	+-	0.051 ±0.042	2.29E-01
cg06786424	*PRPF4B*	0.173 ±0.046	2.55E-04 1.04E-02	0.056 ±0.034	1.06E-01	++	0.097 ±0.027	3.71E-04

This table summarises the ten differentially methylated regions most highly associated with PC2 in the discovery EWAS (27K). In the discovery EWAS (27K) association of DNA methylation was associated with PC2 in a linear mixed effect model adjusted for batch effects, chronological age and subject relatedness. The ten most highly associated probes are characterised by the gene they localise to. The effect (beta), standard error of the effect (se) and significance of model fit (p-value) are reported. To confirm that association was not driven by association with chronological age, significance of model fit excluding age as a model parameter (p-value (no age)) was added. The ten most highly associated probes were taken forward for replication in an independent sample of 203 subjects (n=203) with 450K DNA methylation bead chip data available (450K). The effect (beta) standard error of the effect and significance of model fit (p-value) in the replication sample (450K) are reported. The associations reported for the discovery EWAS (27K) and replication (450K) sample were meta analysed (meta-analysis) using an inverse variance scheme. The direction of effect for both samples (dir), combined effect (beta), standard error of the combined effect (se) and significance of the combined association (p-value) are listed for the meta-analysis.

Replication of top EWAs findings in an independent replication sample

The ten most highly associated CpG probes determined in the discovery sample were taken forward for replication in an independent sample of 203 females from the TwinsUK cohort with available pure-tone audiometry and Illumina Infinium 450K Bead chip data (Table 39, replication). In agreement with the replication criteria defined above association between DNA methylation and hearing PC1 was replicated for probes cg01161216 ($p(450K)=8.55x10^{-5}$) and cg18877514 ($p(450K)=1.70x10^{-2}$) (Table 40), mapping to *TCF25* and *POLE*, respectively. The association between hearing PC1 and DNA methylation residuals (adjusted for age, batch effects and relatedness) for the discovery and the replication cohort at cg01161216 and cg18877514 is depicted in Figure 45. Additionally, 5 out of the 10 most highly associated DMRs for PC1 showed the same direction of effect in the replication sample (cg25383093, cg19923810, cg21370143, cg15791248 and cg25017250), however association in the replication sample did not reach nominal significance for these probes (Table 40). To confirm that association at the replicating probes (cg01161216 and cg18877514) was not driven by association with chronological age, significance of model fit excluding age as a model parameter (Table 40, 450K, p-value (no age)) was determined.

Whether underlying genetic associations might influence the epigenome-wide association signals at cg01161216 and cg18877514 was further investigated. Genome-wide association results from the TwinsUK cohort for age-adjusted hearing PC1 (chapter 5) showed no significant association with SNPs 200kb up- and downstream of the replicating genes (*TCF25* and *POLE*).

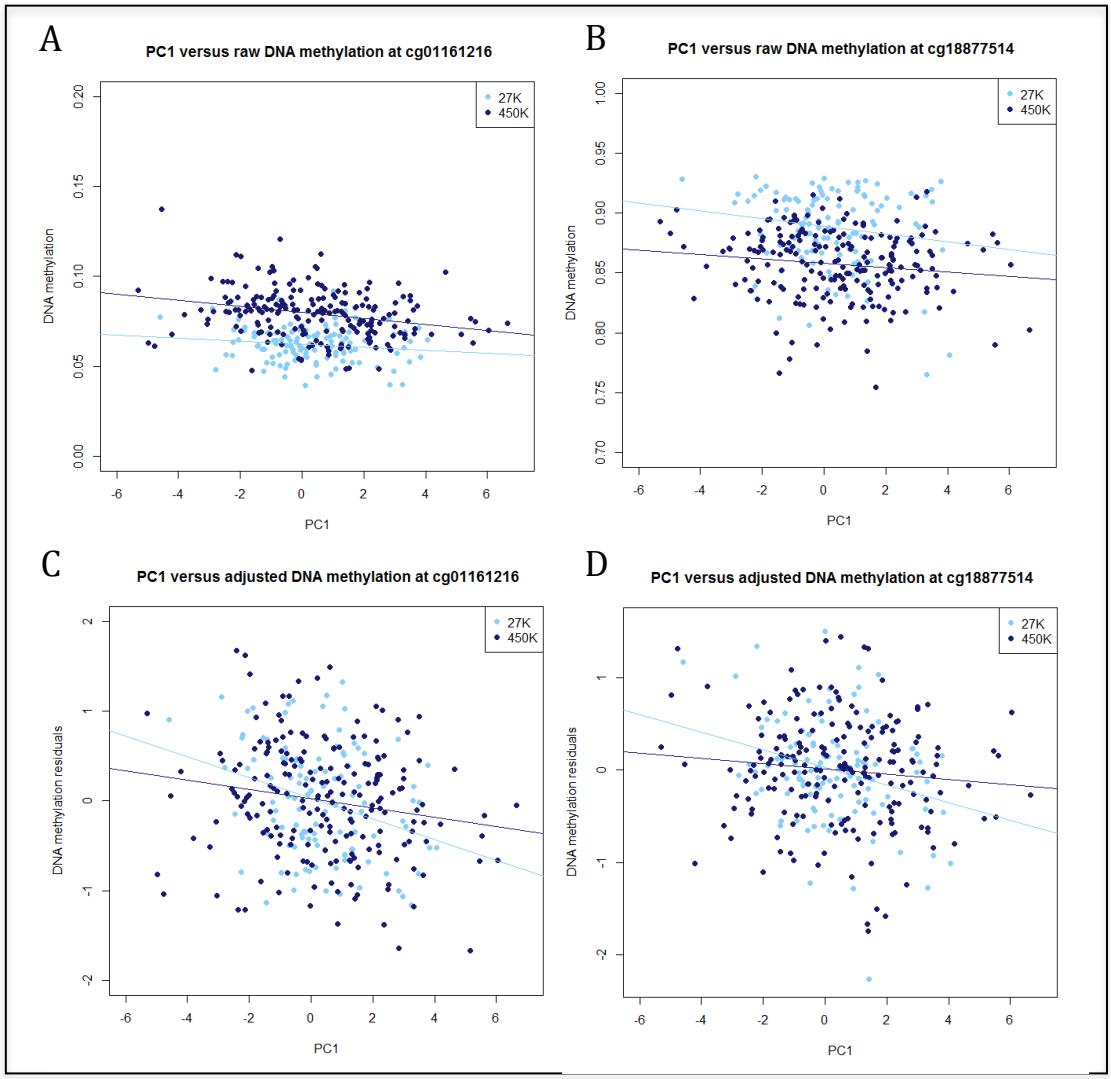


Figure 45 Correlation of hearing PC1 with DNA methylation at cg01161216 (*TCF25*) and cg18877514 (*POLE*)

A, B Hearing PC1 values were plotted versus raw DNA methylation beta (adjusted for age, batch effects and relatedness) for both the discovery (27K, light blue dots) and the replication (450K, dark blue dots) samples. A linear regression lines was fitted for both datasets (27K:light blue line, 450K:dark blue line).
C, D Hearing PC1 values were plotted versus DNA methylation beta residuals (adjusted for age, batch effects and relatedness) for both the discovery (27K, light blue dots) and the replication (450K, dark blue dots) samples. A linear regression lines was fitted for both datasets (27K:light blue line, 450K:dark blue line).

None of the DNA methylation probes associated with PC2 in the discovery EWAS passed replication criteria in the Illumina Infinium Humanmethylation450K dataset. However, 7 out of 10 probes (cg05467918, cg03916787, cg25762395, cg12438037, cg02693857, cg14784653, cg06786424) taken forward for replication showed the same direction of effect observed in the discovery EWAS (Table 41).

Differential DNA methylation at *TCF25* showed the most significant association with PC1 (cg01161216, $p=4.89 \times 10^{-9}$) in the meta-analysis (Table 40). For PC2, the

association with differentially methylated DNA at *MXD3* (cg02693857, $p=1.16x10^{-4}$) was most significant after meta-analysis (Table 41).

Whole Blood heterogeneity

It was tested whether the ten most highly associated probes in the discovery EWAS remained significantly associated with PC1 and PC2 after adjustment for blood cell heterogeneity in a subset of 106 females from the discovery sample (Table 39) with data available. Epigenome-wide association signals for the most highly associated probes in this subset remained significantly associated ($p>0.005$) with PC1 and PC2 after adjustment for blood cell subtype concentrations.

Validation of *TCF25* using MeDIPseq

To validate the peak EWAS DMR using a different technology, TCF25 DNA methylation levels based on MeDIPseq data were also explored for association with hearing in 46 unrelated females from TwinsUK [66]. The mean age of subjects in the validation sample was 62.28 (± 7.86 years of standard deviation from the mean, age range 43 - 86 years) (Table 39, validation). MeDIPseq DNA methylation levels at a 1kb locus overlapping probe cg01161216 were selected and compared to PC1. DNA methylation at this locus was significantly associated with hearing PC1 ($p=4.09x10^{-2}$) and showed the same direction of effect (beta ± se=$-8.72x10^{-6} \pm 4.13x10^{-6}$) as both the discovery EWAS and replication datasets.

Influence of DNA methylation on gene expression

To determine the influence of DNA methylation and hearing measures on gene expression, DNA methylation levels at the replicating probes in *TCF25* and *POLE* (cg01161216, cg18877514) and PC1 values were compared to gene expression levels for the corresponding genes from the MuTHER database [67]. Gene expression data were available for three different tissues in 172 females with corresponding hearing and DNA methylation (27K) measures. It was decided to compare DNA methylation to gene expression in skin tissue, due to its shared embryonic origin with inner ear tissue. DNA methylation residuals and gene expression residuals showed a weak negative correlation with *TCF25* (r=-0.02) (Figure 46, A) and *POLE* (r=-0.06) expression (Figure 46, B). Cytosine DNA methylation in the promoter region of a gene should represses gene expression, thus a negative correlation between DNA methylation and gene expression was expected. In addition, the effect of gene expression of *TCF25* and *POLE* on hearing ability with age was investigated by correlating gene expression residuals for *TCF25* and *POLE* with PC1 values. Expression levels of both genes

showed weak positive correlations with PC1 values (*TCF25*: r=0.12; *POLE*: r=0.16) (Figure 46, C and D).

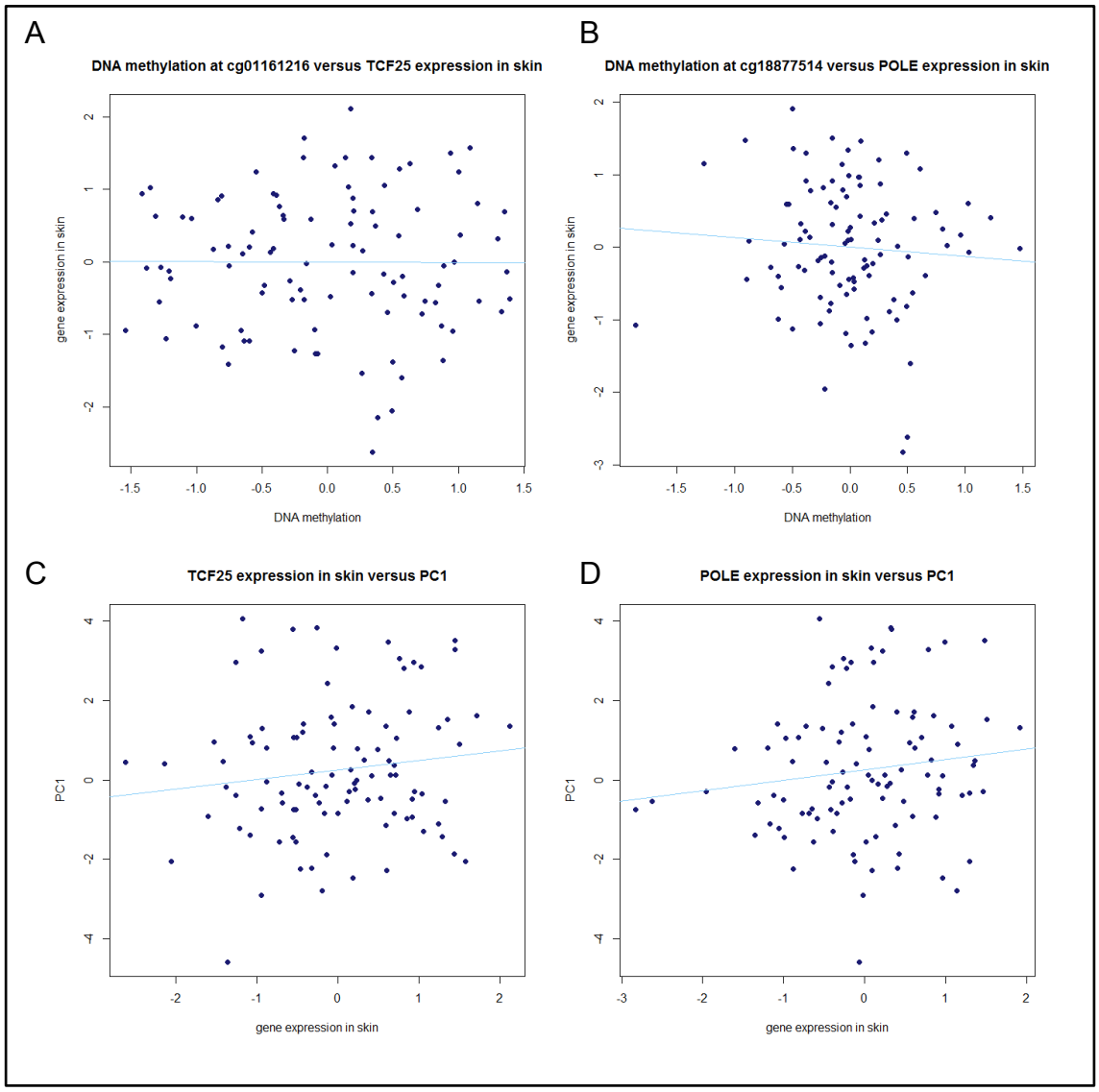


Figure 46 Influence of DNA methylation and hearing ability on gene expression

A. DNA methylation residuals showed a weak negative correlation (r=-0.02) with expression residuals of TCF25 in skin samples. Both quantile normalised DNA methylation betas and quantile normalised gene expression values were adjusted for experimental batch effects (chip and position on the chip for Methylation betas and for gene expression experimental batch and RNA concentration) previous to analysis. The regression line (blue line) depicts the linear association between DNA methylation residuals and gene expression residuals. **B.** DNA methylation residuals at probe cg18877514 were weakly correlated (r=-0.06) with *POLE* expression residuals in skin tissue. Both quantile normalised DNA methylation betas and quantile normalised gene expression values were adjusted for experimental batch effects (chip and position on the chip for Methylation betas and for gene expression experimental batch and RNA concentration) previous to analysis. The regression line (blue line) depicts the linear association between DNA methylation residuals and gene expression residuals. **C.** *TCF25* expression residuals in skin showed a weak negative correlation (r=0.12) with PC1. Quantile normalised gene expression values were adjusted for experimental batch effects and RNA concentration. The regression line (blue line) depicts the linear association between gene expression residuals and PC1 values. **D.** *POLE* expression

residuals in skin showed a weak negative correlation (r=0.16) with PC1. Quantile normalised gene expression values were adjusted for experimental batch effects and RNA concentration. The regression line (blue line) depicts the linear association between gene expression residuals and PC1 values.

Differentially methylated regions in monozygotic twins discordant for hearing

For the MZ co-twin analyses, all MZ twin pairs from the discovery sample (Table1) were selected. This dataset comprised 21 female MZ twin pairs (n=42) with a mean age of 62.00 years ±6.60 years of SD (age range: 50-72 years) (Table 39). In the discordant MZ twin pair analysis, difference in hearing ability within identical twin pairs was correlated with difference in DNA methylation determined between the same twin sisters. Phenotypic mean difference within twin pairs (measured as difference between twin sister 1 and 2) as measured by PC1 was -0.42± 1.34 SD and ranged between -3.47 and 2.86. Phenotypic mean difference within twin pairs as measured by PC2 was -0.21± 1.02 SD and ranged between -1.97 and 1.92. Difference in DNA methylation was measured at 24641 CpG sites genome-wide. Discordance in hearing PC1 was most highly correlated with differential methylation at probe cg01377755, mapping to the promoter of the lysophosphatidic Acid Phosphatase 6 (*ACP6*) gene (r=-0.753, $p=1.24x10^{-4}$). Further strongly correlated differentially methylated genes included Myocyte Enhancer Factor 2D (MEF2D, cg08156349,r=-0.749, $p=1.41x10^{-4}$), Tachykinin Precursor 1 (*TAC1*, cg07550362, r=-0.722, $p=3.25x10^{-4}$), ATPase Family AAA Domain-Containing 3C (*ATAD3C*, cg27383362, r=-0.703, $p=5.49x10^{-4}$), brain-specific Serine Protease 3 (*PRSS12*, cg21208104, r=0.697, $p=6.26x10^{-4}$), ADAM Metallopeptidase Domain 18 (*ADAM18,* cg23566335, r=0.696, $p=6.47x10^{-4}$), Chromobox Homolog 2 (*CBX2*, cg22892904, r=-0.688, $p=7.82x10^{-4}$), Septin 3 (*SEPT3*, cg04283938, r=-0.684, $p=8.59x10^{-4}$), Transmembrane Protein 121 (*TMEM121*, cg23886551, r=-0.684, $p=8.59x10^{-4}$) and Torsin Family 1 Member B (*TOR1B*, cg14299800, r=-0.682, $p=9.13x10^{-4}$) (Table 42 and Appendix Figure 52).

Discordance in hearing PC2 was most strongly correlated with differential methylation at probe cg12113132 in the promoter of the Cyclin D-Type Binding-Protein 1 (*CCNDBP1*) gene (r=-0.792, $p=2.48x10^{-5}$). Further differentially methylated probes in strong correlation with PC2 discordance were determined for the Serine/Arginine-Rich Splicing Factor 6 (*SRSF6*) gene (cg07440387, r=0.752, $p=1.29x10^{-4}$), the Arginine/Serine-Rich Coiled-Coil 2 (*RSRC2*) gene (cg11157872, r=0.751, $p=1.35x10^{-4}$), the G-protein coupled Purinergic Receptor P2Y 12 (*P2RY12*) gene (cg05094216, r=-0.751, $p=1.35x10^{-4}$), the RAB10 member of the RAS Oncogene Family (*RAB10*) gene (cg11109684, r=0.721, $p=3.37x10^{-4}$), the Histone deacetylase 4 (*HDAC4*) gene

(cg19028160, r=0.718, p=3.62x10^{-4}), the Rho-interacting serine/threonine kinase 21 (*CIT*) gene (cg25499017, r=0.717, p=3.76x10-4), the Zinc finger protein 610 (*ZNF610*) gene (cg07703337, r=-0.706, p=4.96x10^{-4}), the tubulin tyrosine ligase-like family member 3 (*TTLL3*) gene (cg14800883, r=0.700, p=5.86x10-4) and the microRNA 638 (*MIR638*) gene (cg10948777, r=0.697, p=6.26x10^{-4}) (Table 42 and Appendix Figure 53).

Table 42 Results of the MZ pair difference analysis

MZ pair difference analysis (n=42)							
PC1				**PC2**			
probe	**gene**	**rho**	**p-value**	**probe**	**gene**	**rho**	**p-value**
cg01377755	*ACP6*	-0.753	1.24E-04	cg12113132	*CCNDBP1*	-0.792	2.48E-05
cg08156349	*MEF2D*	-0.749	1.41E-04	cg07440387	*SRSF6*	0.752	1.29E-04
cg07550362	*TAC1*	-0.722	3.25E-04	cg11157872	*RSRC2*	0.751	1.35E-04
cg27383362	*ATAD3C*	-0.703	5.49E-04	cg05094216	*P2RY12*	-0.751	1.35E-04
cg21208104	*PRSS12*	0.697	6.26E-04	cg11109684	*RAB10*	0.721	3.37E-04
cg23566335	*ADAM18*	0.696	6.47E-04	cg19028160	*HDAC4*	0.718	3.62E-04
cg22892904	*CBX2*	-0.688	7.82E-04	cg25499017	*CIT*	0.717	3.76E-04
cg04283938	*SEPT3*	-0.684	8.59E-04	cg07703337	*ZNF610*	-0.706	4.96E-04
cg23886551	*TMEM121*	-0.684	8.59E-04	cg14800883	*TTLL3*	0.700	5.86E-04
cg14299800	*TOR1B*	-0.682	9.13E-04	cg10948777	*MIR638*	0.697	6.26E-04

This table shows the results of the ten most highly correlated DNA methylation probes with MZ twin pair discordance in PC1 and PC2. Correlation between differences in PC1 and PC2 within twin pairs and differential DNA methylation residuals (adjusted for chip and position on the chip) was assessed using spearman correlation. Results are listed for the ten most highly correlated probes with the corresponding gene, Spearman rank correlation coefficient (rho) and significance of correlation (p-value).

Discussion

Changes in DNA methylation have been associated with increasing age and age-related disorders [40]. Here, the association between genome-wide DNA methylation and hearing ability with age has been demonstrated for the first time. This approach identified epigenetic changes at several genes that associated with hearing ability with age, and replicated in a second independent sample of unrelated subjects. Altogether, a significant association with hearing (PC1, PC2) and DNA methylation in the promoter of *TCF25* and *ACADM* were observed. Further, the associations of hearing PC1 with *TCF25* and *POLE* were replicated in an independent sample. In addition, DNA methylation at *FGFR1* was significantly associated with PC1, a gene known to be

essential for maintenance of glial cells and cochlear neurons in the spiral ganglion [68]. We suggest, that epigenetic changes with age might account for the late age of onset for ARHI and explain a proportion of the missing heritability for this phenotype.

Two differentially methylated regions at *TCF25* and *POLE* were highly associated with hearing PC1 in the discovery EWAS and replicated in the replication study (450K). At both gene promoters individuals with reduced hearing ability (high PC1 value) showed lower DNA methylation levels. The association of these two DMRs with PC1 could neither be explained by blood cell heterogeneity in the venous blood samples nor by underlying genetic variants at the corresponding loci. *TCF25*, also referred to as *NULP1*, belongs to the family of helix-loop-helix transcription factors and was shown to be expressed in mouse dorsal root ganglia during embryonic development [69]. In addition, *TCF25* encodes a protein with a novel transcriptional repressive domain, which can repress the transcription of other genes [70]. *Tcf25* deficient mouse models have not been reported yet, but might shed further light on the function of this gene.

POLE encodes the catalytic subunit of DNA polymerase epsilon, the enzyme responsible for extension of the leading strand during DNA replication [71]. Mutations in *POLE* have been reported in patients with colorectal cancer, likewise mice homozygous for a knock-in allele of *Pole* show increased incidence of various cancers. Mice with a transgenic gene disruption of *Pole* die before birth, highlighting the essential function of this gene.

According to the ENCODE database [72], both cg01161216 and cg18877514 (*TCF25* and *POLE*, respectively) map to regions of the genome with increased enhancer- and promoter-associated histone marks (H3K4Me1 and H3K4Me3) as assessed in various cell lines. Furthermore, ENCODE lists transcription factor binding sites identified by chromatin immunoprecipitation for the loci of both probes. Neither of the two replicating genes has been associated with hearing previously. However, the crucial function of both genes during development and DNA replication, respectively, will require finely regulated gene expression. Disruption of this highly regulated process by a change in DNA methylation in the inner ear, could lead to increased DNA mutation rates and cell death.

DNA methylation levels in both the discovery EWAS and replication study were obtained in methods based on bisulfite conversion and DNA annealing. As proof that our findings were not an artefact of this method, technical validation in a different DNA

measurement method, MeDIPseq, was obtained. MeDIPseq is based on enrichment of methylated DNA segments by immunoprecipitation of methylated DNA [47]. The enriched DNA segments are subsequently processed by next generation sequencing. Methylation levels at *TCF25* as measured by MeDIPseq were nominally significant associated with PC1 ($p=4.09x10^{-2}$), thereby supporting our previous findings.

DNA methylation at gene promoters restricts access of transcription factors and DNA transcription machinery to the underlying nucleotide sequence, thereby repressing gene expression. To test whether DNA methylation at *TCF25* and *POLE* had an effect on expression levels of the respective genes, gene expression and DNA methylation levels in 172 individuals were investigated. DNA methylation at both probes showed weakly negative correlations with gene expression (*TCF25*: r=-0.02, *POLE*: r=-0.06). However, DNA methylation and gene expression were measured from different tissues, which might explain the lack of influence on gene expression.

Further, association between gene expression and the hearing (PC1) was investigated by correlation analysis. PC1 values were positively correlated with gene expression at *TCF25* and *POLE*, indicating that high expression of these genes corresponded with reduced hearing ability. Nevertheless, correlations were relatively weak (r= 0.12-0.16) and gene expression in skin, as presented here, might not best represent expression profiles in the inner ear. These findings should therefore be interpreted with caution.

Although we were unable to replicate association between DNA methylation and hearing (PC1; PC2) with further top associated genes from the discovery EWAS, some of these genes represented validated candidate genes for hearing loss and therefore merit discussion here. *FGFR1* encodes the fibroblast growth factor receptor 1 protein. Chemically induced mutations at the *FGFR1* gene in mice resulted in the mouse model “hush puppy”, which displays defects in inner ear morphology and physiology [73,74]. Of particular interest, “hush puppy” mice present with a decreased number of cochlear outer hair cells and reduced endocochlear potential. Furthermore, a conditional mouse knockout of *Fgfr1* and *Fgfr2* in glial cells of the spiral ganglion resulted in loss of spiral ganglion neurons and associated ARHI [75]. These data support an essential function of *FGFR1* in hearing and fine tuned gene expression regulation of this gene appears to be crucial for hearing ability with age. Although *FGFR1* was not significantly associated with DNA methylation in the replication cohort, the direction of effect remained constant. However, the negative association of PC1 with DNA methylation at *FGFR1*

indicates that good hearing ability with age is associated with high methylation and therefore expression repression of *FGFR1*.

The strongest association between DNA methylation and PC2, representing high frequency hearing loss, was observed for the *ACADM* gene encoding medium-chain acyl-CoA dehydrogenase. Deficiency of this protein results in hypoglycaemia and can lead to sudden death in infants if untreated [76]. Although this gene showed the strongest association in the PC2 discovery EWAS, a direct link to hearing ability is as yet unclear.

The sample presented here was collected from the TwinsUK cohort and therefore included MZ and DZ twin pairs (80% of total samples). MZ twin pairs are assumed to be genetically identical and share an increased proportion of environmental exposures (i.e. time in uterus, family environment) compared to normal siblings or unrelated individuals. They have therefore been suggested as the perfect study sample for epigenetic studies in humans, as differences in epigenetic profiles within twin pairs cannot be biased by genetic differences and only to a reduced proportion by differential environmental exposure [4]. Furthermore, epigenetic differences have been suggested as the cause of MZ twin discordance seen for highly heritable traits [59,77]. Particularly the use of MZ twin siblings in the EWAs and replication sample might have reduced confounding by genetic variability in comparison to a sample formed of unrelated singletons only. However, the increased proportion of twin siblings in the EWAS sample also poses the risk of reduced phenotypic variance, potentially limiting power to detect association with moderate to strong epigenetic effects.

All MZ twin pairs from the EWAS sample (n=21 pairs) were taken forward for an epigenome-wide MZ discordance analysis. In this analysis phenotypic differences within twin pairs (hearing PC1 and PC2) were examined for differences within twin pairs' epigenetic profiles. The most strongly correlated probes for PC1 discordance were found in the promoters of the genes *ACP6* and *MEF2D*. The function of acid phosphatase 6 is yet unknown. However, myocyte enhancer factor 2D is a member of the myocyte enhancer factor family of transcription factors, which are involved in neuronal development and differentiation under regulation of class 2 histone deacetylases. *MEF2D* is expressed in mouse cochlear neurons and sensory cells at P15 and was diminished in IGF knockout mice, which show sensorineural hearing loss [78]. These results indicate *MEF2D* to be an interesting candidate gene for ARHI. Reduced expression of *MEF2D* due to DNA methylation could result in sensorineural

hearing loss, as reported for mouse models [78]. Discordance for PC2 was most strongly correlated with differential methylation at *CCNDBP1. CCNDBP1* represses transcriptional activity of cyclin D1 and has been shown to directly interact with Sirt6 [79], a class 3 histone deacetylase linked to cancer and ageing [80].

This study had several strengths and limitations. Strengths included the identification and replication of differentially methylated probes strongly associated with ARHI. Although the associations reported here failed to reach epigenome-wide significance levels conservatively considering all 24641 "independent" probes, the association between PC1 and *TCF25* reached suggestive significant association thresholds ($p=6.90x10^{-6}$) when considering 14495 independent genes. The 27K chip used here was originally developed to measure DNA methylation genome-wide with a particular focus on genes differentially methylated in cancer. Therefore our EWAS findings for PC1 and PC2, two non-cancer phenotypes, might be biased by the selection of gene promoters covered on this chip. Furthermore, recent research shows that the 70-80% of CpG sites currently covered in whole-genome bisulfite sequencing provide only minor information about Dna methylation due to reduced variability of methylation at these sites [81]. Age and blood cell heterogeneity could be excluded as potential confounders of the hearing DMR results. Limitations of our study included the choice of sample tissue. Despite the knowledge that DNA methylation can be tissue specific, DNA methylation from more accessible whole blood samples was used instead of more appropriate but problematic tissues from the inner ear. The only study investigating epigenetic changes in the human inner ear analysed DNA methylation of candidate genes in tumour tissue from vestibular schwanomas [82]. Nevertheless, recent research on the epigenetics of pain sensitivity demonstrated that epigenetic changes in blood samples mirrored those in brain tissue, suggesting a tissue crossover [66]. Phenotypic variation might have been reduced in the EWAs and replication sample as a result of including of a high proportion of twin pairs, which might limit power to detect association with moderate to strong epigenetic effects. Twin relatedness was adjusted for as a random effect in the EWAS and replication linear mixed effect regression models. Although we were able to show a weak negative correlation between DNA methylation levels and gene expression at *TCF25* and *POLE*, these results were obtained from skin samples, which might not be representative of gene expression in the inner ear. Gene expression as measured in skin tissues was selected as the most suitable tissue from the three tissues available [67] (skin, fat and white blood cells) due to its embryonic relatedness with nervous tissue.

While twin siblings might have not been the optimal study sample in the standard EWAS, we took particular advantage of this special relatedness structure in the MZ co-twin analysis. In the EWAS, the confounding effect of genetic variability between study subjects on DNA methylation could not be eliminated completely. However, the inclusion of MZ twin pairs in this sample might have reduced this confounding genetic variance. In contrast, the MZ co-twin design compares only MZ twin siblings within a twin pair. Differences in DNA methylation due to genetic differences should therefore be prevented in this special twin sample. Here, MZ twin siblings showing stronger discordance in hearing ability would provide greater statistical power to detect significant effects, however, limited by the availability of samples with DNA methylation and hearing data available, the twin sisters studied showed only relatively low levels of discordance. Previous studies reported that associations with DNA methylation measured in venous blood samples might be driven by blood cell heterogeneity [65]. Exact blood cell counts were available for 106 of the 115 EWAS samples. DNA methylation at the ten most significant probes remained significantly associated with PC1 after adjustment for blood cell heterogeneity in the EWAS. This showed that blood cell heterogeneity alone could not account for the associations reported in the EWAS. Finally, this study does not allow assumptions about causal relationships between DNA methylation and ARHI. A longitudinal study design would have been required to determine whether changes in DNA methylation are causal to ARHI or whether processes involved in ARHI cause changes in DNA methylation.

Conclusion

This is the first study to date demonstrating the association between hearing ability (as measured by pure-tone audiometry) and DNA methylation genome-wide. We identified suggestive significant associations with DNA methylation in the promoters of various genes, of which two (*TCF25* and *POLE*) could be replicated in an independent cohort and one (*TCF25*) technically validated using a different methylation detection method. Functional studies will be required to further explore the effect of epigenetic regulation of these genes in ARHI. Despite the relatively small sample presented here, our findings strongly support the association of ARHI with epigenetic modifications like DNA cytosine methylation. Future investigations focussing on different epigenetic modifications and larger sample sizes might explain part of the missing heritability in ARHI and revolutionise the understanding and treatment of this complex disorder. Furthermore, these findings are of fundamental importance not only for hearing loss but also other age-related disorders.

References

1. Wong CCY, Caspi A, Williams B, Craig IW, Houts R, et al. (2010) A longitudinal study of epigenetic variation in twins. Epigenetics 5: 516-526.

2. Nagarajan R, Hogart A, Gwye Y, Martin MR, LaSalle JM (2006) Reduced MeCP2 expression is frequent in autism frontal cortex and correlates with aberrant MECP2 promoter methylation. Epigenetics 1: 172-182.

3. Jones PA, Baylin SB (2002) The fundamental role of epigenetic events in cancer. Nature reviews genetics 3: 415-428.

4. Bell JT, Spector TD (2011) A twin approach to unraveling epigenetics. Trends in Genetics 27: 116-125.

5. Wolber LE, Steves CJ, Spector TD, Williams FMK (2012) Hearing Ability with Age in Northern European Women: A New Web-Based Approach to Genetic Studies. PLoS One 7: e35500.

6. Fraga MF, Ballestar E, Paz MF, Ropero S, Setien F, et al. (2005) Epigenetic differences arise during the lifetime of monozygotic twins. Proceedings of the National Academy of Sciences of the United States of America 102: 10604-10609.

7. Provenzano MJ, Domann FE (2007) A role for epigenetics in hearing: Establishment and maintenance of auditory specific gene expression patterns. Hearing Research 233: 1-13.

8. Friedman L, Avraham K (2009) MicroRNAs and epigenetic regulation in the mammalian inner ear: implications for deafness. Mammalian Genome 20: 581-603.

9. Spector T (2012) Identically different: why you can change your genes: Hachette UK.

10. Waddington CH (2012) The Epigenotype. International Journal of Epidemiology 41: 10-13.

11. IUM T (2012) ENCODE project writes eulogy for junk DNA.

12. Russo VE, Martienssen RA, Riggs AD (1996) Epigenetic mechanisms of gene regulation: Cold Spring Harbor Laboratory Press.

13. Bird A (2007) Perceptions of epigenetics. Nature 447: 396-398.

14. Jones PA, Taylor SM (1980) Cellular differentiation, cytidine analogs and DNA methylation. Cell 20: 85-93.

15. Reik W (2007) Stability and flexibility of epigenetic gene regulation in mammalian development. Nature 447: 425-432.

16. Rakyan VK, Down TA, Balding DJ, Beck S (2011) Epigenome-wide association studies for common human diseases. Nat Rev Genet 12: 529-541.

17. Gardiner-Garden M, Frommer M (1987) CpG Islands in vertebrate genomes. Journal of Molecular Biology 196: 261-282.

18. Ehrlich M, Gama-Sosa MA, Huang L-H, Midgett RM, Kuo KC, et al. (1982) Amount and distribution of 5-methylcytosine in human DNA from different types of tissues or cells. Nucleic Acids Research 10: 2709-2721.

19. Fuks F, Hurd PJ, Wolf D, Nan X, Bird AP, et al. (2003) The methyl-CpG-binding protein MeCP2 links DNA methylation to histone methylation. Journal of Biological Chemistry 278: 4035-4040.

20. Baylin SB, Herman JG (2000) DNA hypermethylation in tumorigenesis: epigenetics joins genetics. Trends in Genetics 16: 168-174.

21. Chen RZ, Pettersson U, Beard C, Jackson-Grusby L, Jaenisch R (1998) DNA hypomethylation leads to elevated mutation rates. Nature 395: 89-93.

22. Eden A, Gaudet F, Waghmare A, Jaenisch R (2003) Chromosomal instability and tumors promoted by DNA hypomethylation. Science 300: 455-455.

23. Bird A (2002) DNA methylation patterns and epigenetic memory. Genes & development 16: 6-21.

24. Meissner A, Mikkelsen TS, Gu H, Wernig M, Hanna J, et al. (2008) Genome-scale DNA methylation maps of pluripotent and differentiated cells. Nature 454: 766-770.

25. Bestor TH (2000) The DNA methyltransferases of mammals. Human Molecular Genetics 9: 2395-2402.

26. Schermelleh L, Haemmer A, Spada F, Rösing N, Meilinger D, et al. (2007) Dynamics of Dnmt1 interaction with the replication machinery and its role in postreplicative maintenance of DNA methylation. Nucleic Acids Research 35: 4301-4312.

27. Okano M, Bell DW, Haber DA, Li E (1999) DNA Methyltransferases Dnmt3a and Dnmt3b Are Essential for De Novo Methylation and Mammalian Development. Cell 99: 247-257.

28. Reik W, Walter J (2001) Genomic imprinting: parental influence on the genome. Nat Rev Genet 2: 21-32.

29. Kim J, Samaranayake M, Pradhan S (2009) Epigenetic mechanisms in mammals. Cellular and Molecular Life Sciences 66: 596-612.

30. Okano M, Xie S, Li E (1998) Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. Nature Genetics 19: 219-220.

31. Reik W, Dean W, Walter J (2001) Epigenetic Reprogramming in Mammalian Development. Science 293: 1089-1093.

32. Daxinger L, Whitelaw E (2012) Understanding transgenerational epigenetic inheritance via the gametes in mammals. Nat Rev Genet 13: 153-162.

33. Morgan HD, Sutherland HGE, Martin DIK, Whitelaw E (1999) Epigenetic inheritance at the agouti locus in the mouse. Nature Genetics 23: 314-318.

34. Sinclair KD, Allegrucci C, Singh R, Gardner DS, Sebastian S, et al. (2007) DNA methylation, insulin resistance, and blood pressure in offspring determined by maternal periconceptional B vitamin and methionine status. Proceedings of the National Academy of Sciences 104: 19351-19356.

35. Feil R, Fraga MF (2012) Epigenetics and the environment: emerging patterns and implications. Nature reviews genetics 13: 97-109.

36. Heijmans BT, Tobi EW, Stein AD, Putter H, Blauw GJ, et al. (2008) Persistent epigenetic differences associated with prenatal exposure to famine in humans. Proceedings of the National Academy of Sciences 105: 17046-17049.

37. Kaminsky ZA, Tang T, Wang S-C, Ptak C, Oh GH, et al. (2009) DNA methylation profiles in monozygotic and dizygotic twins. Nature Genetics 41: 240-245.

38. Boks MP, Derks EM, Weisenberger DJ, Strengman E, Janson E, et al. (2009) The Relationship of DNA Methylation with Age, Gender and Genotype in Twins and Healthy Controls. PLoS One 4: e6767.

39. Kerkel K, Spadola A, Yuan E, Kosek J, Jiang L, et al. (2008) Genomic surveys by methylation-sensitive SNP analysis identify sequence-dependent allele-specific DNA methylation. Nat Genet 40: 904-908.

40. Bell JT, Tsai P-C, Yang T-P, Pidsley R, Nisbet J, et al. (2012) Epigenome-Wide Scans Identify Differentially Methylated Regions for Age and Age-Related Phenotypes in a Healthy Ageing Population. PLoS Genet 8: e1002629.

41. Laird PW (2010) Principles and challenges of genome-wide DNA methylation analysis. Nat Rev Genet 11: 191-203.

42. Rakyan VK, Down TA, Maslau S, Andrew T, Yang T-P, et al. (2010) Human aging-associated DNA hypermethylation occurs preferentially at bivalent chromatin domains. Genome Research 20: 434-439.

43. Bibikova M, Le J, Barnes B, Saedinia-Melnyk S, Zhou L, et al. (2009) Genome-wide DNA methylation profiling using Infinium® assay. Epigenomics 1: 177-200.

44. Bibikova M, Barnes B, Tsan C, Ho V, Klotzle B, et al. (2011) High density DNA methylation array with single CpG site resolution. Genomics 98: 288-295.

45. Bock C, Walter J, Paulsen M, Lengauer T (2007) CpG island mapping by epigenome prediction. PLoS computational biology 3: e110.

46. Weber M, Davies JJ, Wittig D, Oakeley EJ, Haase M, et al. (2005) Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. Nat Genet 37: 853-862.

47. Down TA, Rakyan VK, Turner DJ, Flicek P, Li H, et al. (2008) A Bayesian deconvolution strategy for immunoprecipitation-based DNA methylome analysis. Nat Biotech 26: 779-785.

48. Friedman RA, Van Laer L, Huentelman MJ, Sheth SS, Van Eyken E, et al. (2009) GRM7 variants confer susceptibility to age-related hearing impairment. Hum Mol Genet 18: 785-796.

49. Van Laer L, Huyghe JR, Hannula S, Van Eyken E, Stephan DA, et al. (2010) A genome-wide association study for age-related hearing impairment in the Saami. European Journal of Human Genetics 18: 685-693.

50. Van Laer L, Van Eyken E, Fransen E, Huyghe JR, Topsakal V, et al. (2008) The grainyhead like 2 gene (GRHL2), alias TFCP2L3, is associated with age-related hearing impairment. Hum Mol Genet 17: 159-169.

51. Unal M, Tamer L, Dogruer ZN, Yildirim H, Vayisoglu Y, et al. (2005) N-acetyltransferase 2 gene polymorphism and presbycusis. Laryngoscope 115: 2238-2241.

52. Huyghe JR, Van Laer L, Hendrickx JJ, Fransen E, Demeester K, et al. (2008) Genome-wide SNP-based linkage scan identifies a locus on 8q24 for an age-related hearing impairment trait. Am J Hum Genet 83: 401-407.

53. Cruickshanks KJ, Wiley TL, Tweed TS, Klein BE, Klein R, et al. (1998) Prevalence of hearing loss in older adults in Beaver Dam, Wisconsin. The Epidemiology of Hearing Loss Study. Am J Epidemiol 148: 879-886.

54. Fransen E, Lemkens N, Laer LV, Camp GV (2003) Age-related hearing impairment (ARHI): environmental risk factors and genetic prospects. Experimental Gerontology 38: 353-359.

55. Davis RR, Newlander JK, Ling X-B, Cortopassi GA, Krieg EF, et al. (2001) Genetic basis for susceptibility to noise-induced hearing loss in mice. Hearing Research 155: 82-90.

56. Riedemann L, Lanvers C, Deuster D, Peters U, Boos J, et al. (2008) Megalin genetic polymorphisms and individual sensitivity to the ototoxic effect of cisplatin. The pharmacogenomics journal 8: 23-28.

57. Peters U, Preisler-Adams S, Hebeisen A, Hahn M, Seifert E, et al. (2000) Glutathione S-transferase genetic polymorphisms and individual sensitivity to the ototoxic effect of cisplatin. Anti-cancer drugs 11: 639-643.

58. Mastroeni D, McKee A, Grover A, Rogers J, Coleman PD (2009) Epigenetic Differences in Cortical Neurons from a Pair of Monozygotic Twins Discordant for Alzheimer's Disease. PLoS One 4: e6617.

59. Petronis A, Gottesman II, Kan P, Kennedy JL, Basile VS, et al. (2003) Monozygotic twins exhibit numerous epigenetic differences: clues to twin discordance? Schizophrenia bulletin 29: 169-178.

60. Manolio TA, Collins FS, Cox NJ, Goldstein DB, Hindorff LA, et al. (2009) Finding the missing heritability of complex diseases. Nature 461: 747-753.

61. Wilkin DJ, Liberfarb R, Davis J, Levy HP, Cole WG, et al. (2000) Rapid determination of COL2A1 mutations in individuals with Stickler syndrome: Analysis of potential premature termination codons. American Journal of Medical Genetics 94: 141-148.

62. Buschdorf J, Strätling W (2004) A WW domain binding region in methyl-CpG-binding protein MeCP2: impact on Rett syndrome. Journal of Molecular Medicine 82: 135-143.

63. Bell JT, Pai AA, Pickrell JK, Gaffney DJ, Pique-Regi R, et al. (2011) DNA methylation patterns associate with genetic and gene expression variation in HapMap cell lines. Genome Biol 12: R10.

64. Willer CJ, Li Y, Abecasis GR (2010) METAL: fast and efficient meta-analysis of genomewide association scans. Bioinformatics 26: 2190-2191.

65. Horvath S, Zhang Y, Langfelder P, Kahn RS, Boks MP, et al. (2012) Aging effects on DNA methylation modules in human brain and blood tissue. Genome Biol 13: R97.

66. Bell JT, Loomis AK, Butcher LM, Gao F, Zhang B, et al. (2014) Differential methylation of the TRPA1 promoter in pain sensitivity. Nat Commun 5.

67. Grundberg E, Small KS, Hedman AK, Nica AC, Buil A, et al. (2012) Mapping cis- and trans-regulatory effects across multiple tissues in twins. Nat Genet 44: 1084-1089.

68. Wang SJ, Furusho M, D'Sa C, Kuwada S, Conti L, et al. (2009) Inactivation of fibroblast growth factor receptor signaling in myelinating glial cells results in

significant loss of adult spiral ganglion neurons accompanied by age-related hearing impairment. Journal of Neuroscience Research 87: 3428-3437.

69. Olsson M, Durbeej M, Ekblom P, Hjalt T (2002) Nulp1, a novel basic helix-loop-helix protein expressed broadly during early embryonic organogenesis and prominently in developing dorsal root ganglia. Cell and Tissue Research 308: 361-370.

70. Cai Z, Wang Y, Yu W, Xiao J, Li Y, et al. (2006) hnulp1, a basic helix-loop-helix protein with a novel transcriptional repressive domain, inhibits transcriptional activity of serum response factor. Biochemical and Biophysical Research Communications 343: 973-981.

71. Budd ME, Campbell JL (1993) DNA polymerases delta and epsilon are required for chromosomal replication in Saccharomyces cerevisiae. Molecular and Cellular Biology 13: 496-505.

72. Rosenbloom KR, Sloan CA, Malladi VS, Dreszer TR, Learned K, et al. (2013) ENCODE Data in the UCSC Genome Browser: year 5 update. Nucleic Acids Research 41: D56-D63.

73. Pau H, Fuchs H, de Angelis MH, Steel KP (2005) Hush puppy: a new mouse mutant with pinna, ossicle, and inner ear defects. The Laryngoscope 115: 116-124.

74. Calvert J, Dedos S, Hawker K, Fleming M, Lewis M, et al. (2011) A missense mutation in Fgfr1 causes ear and skull defects in hush puppy mice. Mammalian Genome 22: 290-305.

75. Wang S, Furusho M, D'sa C, Kuwada S, Conti L, et al. (2009) Inactivation of fibroblast growth factor receptor signaling in myelinating glial cells results in significant loss of adult spiral ganglion neurons accompanied by age□related hearing impairment. Journal of Neuroscience Research 87: 3428-3437.

76. Kølvraa S, Gregersen N, Christensen E, Hobolth N (1982) In vitro fibroblast studies in a patient with C6-C10-dicarboxylic aciduria: evidence for a defect in general acyl-CoA dehydrogenase. Clinica Chimica Acta 126: 53-67.

77. Poulsen P, Esteller M, Vaag A, Fraga MF (2007) The epigenetic basis of twin discordance in age-related diseases. Pediatric research 61: 38R-42R.

78. Sanchez-Calderon H, Rodriguez-de La Rosa L, Milo M, Pichel JG, Holley M, et al. (2010) RNA microarray analysis in prenatal mouse cochlea reveals novel IGF-I target genes: implication of MEF2 and FOXM1 transcription factors. PLoS One 5: e8699.

79. Ma W, Stafford LJ, Li D, Luo J, Li X, et al. (2007) GCIP/CCNDBP1, a helix–loop–helix protein, suppresses tumorigenesis. Journal of cellular biochemistry 100: 1376-1386.

80. Denu JM, Gottesfeld JM (2012) Minireview Series on Sirtuins: From Biochemistry to Health and Disease. Journal of Biological Chemistry 287: 42417-42418.

81. Ziller MJ, Gu H, Muller F, Donaghey J, Tsai LTY, et al. (2013) Charting a dynamic DNA methylation landscape of the human genome. Nature 500: 477-481.

82. Lassaletta L, Bello MJ, Del Río L, Alfonso C, Roda JM, et al. (2006) DNA methylation of multiple genes in vestibular schwannoma: relationship with clinical and radiological findings. Otology & Neurotology 27: 1180-1185.

Chapter 8: Discussion

Age related hearing impairment (ARHI) represents the loss of hearing ability acquired with increased age. Action on Hearing Loss has reported that one sixth of the UK population (~10,130,000 individuals) is affected by HL, with 41.7% of all adults aged 50 years or older showing some form of hearing loss [1], making it a common disorder in the elderly. Loss of hearing ability can have significant effects on social interaction [2], and result in loss of physical and mental wellbeing as well as incapacity to work. Particularly in ageing Western populations, the prevalence of this and other ageing traits is likely to rise with the predicted shift in population structure.

Early studies of ARHI described 4 different pathologies: sensory, neural, strial and mechanical ARHI [3], with sensory presbycusis presenting the most commonly described form of ARHI seen in 50% of studied subjects. Sensorineural ARHI is defined by a loss of sensory hair cells particularly in the basal part of the cochlea, which is further associated with damage to supportive cells and spiral ganglion neurons. However, the neuronal damage was suspected to represent a secondary effect to the loss of supportive cells and hair cells.

Despite the high prevalence and impact of ARHI on everyday life, the pathological processes underlying this condition are still poorly understood. Furthermore, once manifested, this loss of hearing ability is not reversible and likely to progress with limited management options available. Current management options focus primarily on the fitting of digital hearing aids. Cochlear implants could provide a good treatment option for the future, but are currently neither cost nor risk efficient to be used routinely, and pharmaceutical treatments have yet to be developed. Better understanding of the causative factors in ARHI might improve the treatment and prevention of this common age-related trait.

The aim of this study was to better understand the factors leading to ARHI in previously healthy individuals. Epidemiological data was collected from mature to elderly females from the United Kingdom. To measure ARHI two hearing tests were to be used: standard pure-tone audiometry and a novel web-based speech-in-noise test, with the new test being compared to and validated against the existing gold standard. The proportion of variance in hearing ability explained by genetic and environmental risk factors was to be estimated based on the classical twin study design. The specific

focus of this study was to identify common genetic variants and epigenetic modifications associated with hearing ability with age. Despite moderate to high heritability estimates of ARHI determined in previous studies, genetic investigations had yet failed to explain this heritability.

It was decided to study females for two reasons. Firstly, the TwinsUK sample historically has a preponderance of female subjects. Also, previous studies of ARHI focussed primarily on male or mixed gender cohorts [4,5,6,7], where ARHI severity is more pronounced in men, showing an earlier age of onset than in women [8]. Hearing ability was measured in 1309 females from the TwinsUK registry using pure-tone audiometry and a customized hearing questionnaire covering exposure to known medical and environmental risk factors of ARHI.

Data collection and phenotype

Pure-tone audiometry is accepted as the gold standard hearing test by most audiologists and aims to determine the lowest sound intensity an individual can hear for different frequencies within the human hearing spectrum. Measured frequencies usually range from 0.25 to 8 kHz with hearing ability at these frequencies being determined for both ears separately. Standard procedures for air-and bone-conduction pure-tone audiometry with and without masking have been published by the British Society of Audiology [9]. While pure-tone audiometry provides detailed information about an individual's hearing ability, using all collected pure-tone thresholds (PTTs) would generate a multitude of data. Previous studies of ARHI divided study participants into cases and controls depending on hearing ability [10] or recruited individuals with particularly extreme age-related hearing phenotypes [6] to increase effect size and therefore statistical power. In this study, we chose to measure hearing ability as a continuous trait rather than comparing affected ("cases") and unaffected ("controls") individuals and thereby retaining maximal hearing information and sample. Due to the choice of measuring ARHI in the population, only one third of the sample met the diagnostic criteria for mild to moderate hearing loss. It might therefore be inappropriate to refer to the phenotype under study as ARHI. Accordingly, the phenotype was referred to as hearing ability with age, rather than ARHI.

There were limitations to our data collection, like the lack of bone conduction testing and a sub-optimal testing environment. To limit their influence subjects with reported forms of conductive HL were excluded from the analysis. In addition, ambient noise

levels were monitored on a regular basis to ensure compliance with BSA standards [9]. Despite these limitations, test results were highly repeatable with a mean difference of ΔPTA=2.082 dB HL within subjects having performed the test twice with a mean difference of 16 months between both test dates. According to previous research [11] in the Beaver Dam cohort the odds of hearing loss increases by a factor of 1.88 for every 5 years of age (OR=1.88, 95% CI:1.80-1.97). Furthermore, the noticed change of 2 dB HL over 16 months appeared low compared to the PTA standard deviation from the mean (SD=10.3207) measured for all 1309 particpants (chapter 2, Table 4), ie only 0.2 SD.

Twins were chosen as the study sample. Twins in general and from the TwinsUK cohort in particular have been shown to be comparable with the general singleton population in cardiovascular mortality [12] and other continuous traits [13], respectively. This supports their suitability in studying age related traits. Same gender twins are of great advantage in heritability studies [14,15], which dissect phenotypic variance into genetically and environmentally determined variance, but less so in genome-wide association studies, where the lack of genetic variance within monozygotic twin siblings reduces total variance and decreases statistical power for the study sample.

Various summary measures of PTTs have been created which are used to varying degrees in current research of ARHI. Severity of hearing difficulty has traditionally been measured by averaging measured PTTs over various pitches [16], referred to as pure-tone average (PTA) or better ear hearing threshold level (BEHL)[17]. Further summary methods included the calculation of principal components[18] or standardised Z-scores [19].

In accordance with the pathology described by *Schuknecht and Gacek* [3] sensorineural ARHI manifests initially in the higher frequencies of the human hearing spectrum and progresses eventually to the medium and lower frequencies, thereby causing a characteristic downslope in the audiogram for the higher frequencies (>2 kHz). We considered the different pure-tone audiometry summary methods listed above in their suitability to best measure this characteristic HL in ARHI. While averaging methods like the PTA and BEHL do not provide important information about the shape of the audiogram, standardised hearing Z-scores only reflect standard hearing ability in subjects below the age of 70 years. Principal components (PCs) do reflect the shape of the audiogram and can be calculated for all age groups. It was thus decided to use PCs as the main measure of hearing ability in this study of ARHI.

Together, the first two PCs explained 70.3%-76.0% of variance in PTTs in the female TwinsUK samples (n=1309). In keeping with previous reports [18], PC1 was found to represent the horizontal threshold shift in PTTs (previously referred to as “magnitude” [18]), while PC2 reflected the slope of the audiogram. Using principal component analysis two major shape features of the audiogram could thus be measured without having to separate samples into subgroups of specific audiometric shapes as done previously [8,20,21,22] and thereby reduce sample size for the different subsamples. A comparison of mean PTTs for groups of TwinsUK volunteers showing low, medium or high PC1 or PC2 values, respectively, revealed that a high PC1 value corresponded with raised PTTs over all frequencies and a high PC2 value corresponded to a high frequency sloping HL. Despite their advantages in measuring hearing ability in respect to ARHI, PCs are not measured on a decibel scale and thus more difficult to interpret. Furthermore, PC values are specific for the hearing cohort calculated in and would need to be standardised (i.e. by rank- transformation) before comparing values between cohorts.

Hearing ability in TwinsUK females was generally better than in samples of comparable gender, ethnicity and age-range [17], with 33.60% of our participants showing at least a mild hearing difficulty (PTA≥25 dB HL). This observation might underlie a general selection bias resulting from the voluntary recruitment strategy used by the TwinsUK register, which favoured more mobile and thus healthier volunteers, who could attend our research facilities at St. Thomas hospital, London. However, the cohort has been shown to be representative of the UK singleton population in various medical and demographic measures [13] as well as for genetic factors [23,24,25].other population based studies of ARHI often recruit subjects passed on subjects registered in a specific residential area [8,26]. In addition, it should be mentioned that we rated HL according to the grades of hearing impairment published by the WHO [16] (mild HL: PTA>25 dB HL), while hearing descriptors as suggested by the British Society of Audiology would have been more stringent (mild HL: PTA>20 dB HL) [9], increasing the prevalence of mild HL.

As seen for other age related traits, variation in hearing ability in our sample increased with age. Previous studies reported ARHI to be associated with exposure to environmental risk factors including noise exposure, gender, smoking and alcohol consumption and cardiovascular diseases [4,5,27,28,29]. Association with environmental risk factors (otitis media during childhood, noisy handiwork and occupational noise exposure) in the females presented here explained only a minor

fraction of variance in PC1 and PC2 (2-3% of variance explained, respectively). This is likely due to the generally low exposure of these subjects to previously determined environmental risk factors.

Heritability study

Previous heritability studies in the elderly have shown variance in hearing detection thresholds to be determined by both genetic and environmental factors, with heritability estimates for ARHI varying widely between 25-100% [8,30]. In addition, heritability estimates of ARHI have shown differences between genders (heritability estimates: males= 47.4- 58.4%; females= 75% (95% CI: 67- 81) [17,30]). A similar gender bias has been observed for other traits [31], indicating perhaps different underlying aetiologies or ages of onset for both sexes. Heritability analyses of ARHI in the studied samples showed a moderate heritability for ARHI (A%=56-61). In general, the AE model, taking into account additive genetic effects and environmental exposure unshared within twin siblings, provided the best model fit under the principle of parsimony. This suggests that shared environmental factors play only a minor role in hearing ability at this age (age range: 40-86 years, mean age± SD: 61.64±8.48). Slightly higher heritability estimates (A=75% (95% CI: 67-81)) had been determined for a similar Finnish twin cohort (100% female; age range: 63-76 years) [17], supporting our findings. The increased heritability estimates seen in the Finnish twin sample might be influenced by reduced phenotypic variance, limited environmental exposure to risk factors for hearing loss and the rather narrow age range of this sample (age range: 63-76 years).

In TwinsUK, the heritability of hearing ability as measured by PC1 and PC2 increased with advanced age (ΔA%=9-20). This increase in heritability was positively correlated with an increase in phenotypic variance observed for the higher age groups. In contrast, a study on heritability of ARHI in male twins reported an increased effect of environmental exposure for higher age groups [30]. The difference in results might be influenced by the increased noise exposure (both occupational and recreational) and differences in underlying aetiology between the two genders. The observation that heritability estimates increased with age could be used to determine the optimal age at which heritability would be maximised and incorporate this into future recruitment strategies for genetic association studies of this trait. Unfortunately, this was not possible in the current study due to limited sample size in the highest age group (age range: 66-86 years), but should be taken into consideration for future studies of ARHI.

The increase in heritability with increasing age as reported here goes against the theory of a cumulative effect of environmental exposure on hearing impairment. Similar results have been found for cognitive function. Early research showed that genetic effects had a greater impact on cognitive function in the elderly [32]. However, longitudinally studies of age related cognitive function in the very elderly population (>70 years) showed that the change in cognitive function within subjects was increasingly influenced by environmental factors [33,34]. This change in heritability in the oldest of the old is further reflected in the low heritability (h^2=23-26%) measured for age of death [35]. Due to the lack of very elderly subjects (age range: 40-86 years) and longitudinal hearing data in this sample the change in heritability for very elderly subjects could not be determined, but would be interesting to investigate in future analyses.

Web-based speech-in-noise phenotype

The common disease common variant hypothesis proposes that complex diseases with a moderate to high prevalence in the population might be caused by genetic variants equally common in the population. In line with the moderate heritability estimates in TwinsUK, it was decided to test for an association between genotype and hearing ability with age in a genome-wide association study. Previous studies aimed at determining causative genetic variants in ARHI have yet failed to reach genome-wide significance thresholds [6,10,18,36,37]. This might be due to the relatively low sample sizes used in each of these studies. Pure-tone audiometry is the accepted gold standard hearing test to determine a hearing loss, however, the test procedure may be relatively lengthy and requires specialised personal and equipment to administer the test. This naturally limits the number of samples that can be collected for research studies in a given timeframe. A quick hearing check that could be performed from at home and gave a direct measure of hearing might facilitate a faster collection of larger datasets and help to recruit less mobile subjects.

Speech-in-noise test validation

A web based hearing test developed to measure speech perception in background noise [38,39,40] and kindly provided for this research by Action on Hearing Loss (http://www.actiononhearingloss.org.uk) was chosen to collect hearing data that might be used to capture ARHI for future GWAS. Difficulty understanding speech in a noisy environment (i.e. in a group of subjects or with the TV on in the background) is one of the first symptoms of ARHI reported by most individuals. To determine the suitability of this novel test to correctly diagnose ARHI, sensitivity and specificity to diagnose moderate hearing loss (defined as a PTA≥40 dB HL) was investigated in 448 subjects

having completed both pure-tone audiometry and the web based test. The web based hearing test showed 88.24% sensitivity and 80.05% specificity at a speech reception threshold (SRT) of -9.25 dB to correctly identify moderate HL in our sample. Despite the moderate sensitivity and specificity determined for the speech in noise test, it should be considered that both hearing tests (pure-tone audiometry and speech-perception in noise) might measure different aspects of hearing (namely pitch perception in quiet and speech perception in noise). However, it should be mentioned that speech perception difficulties represent a symptom of ARHI and might be influenced by different pathologies than pitch perception as measured in the audiogram. For example individuals diagnosed with auditory neuropathy might present with normal pure-tone detection thresholds but strong speech-perception impairment. In addition, access to this test via the world-wide-web might have prevented less technically advanced subjects from participating in this part of the study. In conclusion, the speech-in-noise test presents a good surrogate for pure-tone audiometry to collect large datasets for hearing studies. However, subjects being diagnosed with hearing loss according to their SRT value should seek further hearing assessment using pure-tone audiometry.

Bivariate Heritability

Heritability estimates for speech perception in noise were significantly lower than heritability estimates for PC1 and PC2. This might indicate that ability to understand speech in noise might be strongly influenced by environmental exposure, while a reduction in pure-tone perception with age is more strongly determined by genetic make up. It would have been interesting to test for association between speech perception in noise and exposure to environmental risk factors (i.e. noise exposure), however, information on environmental exposure was only available for a minority of subjects having completed the web based test. Nevertheless, this possible association might be further investigated in future studies.

Due to the moderate (~80%) specificity and sensitivity of the web based test to diagnose hearing loss (PTA≥40 dB HL), it was hypothesized that variance in tests measuring the same trait might be explained by shared genetic factors. To determine the extent of this shared variance, a bivariate heritability analysis was conducted. Genetic correlations between PTAs and SRTs ranged from r=-0.7 and r=-0.4 and explained 68.8% and 36.8% of phenotypic correlation between both measures. Similar findings were reported for a Finnish twin sample (100% female; age range: 63-76 years), which measured hearing levels as BEHLs and better ear speech recognition threshold levels (BESRLs). BESRLs and BEHLs shared a genetic component, which

explained 54% (95% CI: 43-64) of the variance in BESRLs. Nevertheless, comparison with our results was limited by the differences in hearing measures, the different choice of bivariate heritability models and samples between both studies.

Having determined a shared genetic component between measures of pitch perception and speech perception in noise, we decided to investigate genes associated with the SRT as an approach to better understand ARHI. It was decided that both SRTs and PCs could both be used as hearing measures for genome-wide association studies (GWASs) of ARHI. Using both phenotypes might further increase sample size and power to detect significant associations. Alternatively, samples with either SRT or PC measures of hearing might serve as replication samples for each other in GWASs. While both tests likely measure different aspects of hearing loss they might also complement each other.

Genome-wide association studies of hearing ability with age

A linear mixed effect regression was applied to determine genetic variants significantly associated with hearing ability in mature female subjects of the TwinsUK registry. Four continuous hearing traits were investigated: PC1 (representing the magnitude of the audiogram), PC2 (reflecting the audiogram's slope), the PTA and speech in noise perception as determined in the web-based test. To determine whether genetic variants each of low effect size might cluster in specific genes, an additional gene, rather than SNP, based association study was performed based on the original GWAS results. In addition, it was investigated whether the genetic associations determined in the GWAS were enriched for specific gene ontologies. Significant gene enrichment (False discovery rate≤ 0.05) was observed for ephrin receptors, cell-cell interactions and gene silencing ontologies.

No genome-wide significant ($p \leq 5x10^{-8}$) associations were found. This is a common phenomenon in the study of common traits, which are assumed to be caused by multiple common genetic variants most likely with small or modest effect sizes on the trait [41]. Current genotyping chips are designed to cover tagging SNPs giving maximal haplotype information according to the HapMap project. GWAs rely on this fact that associated tagging SNPs are in LD with the causal variants. Rare causal variants with potentially large effect sizes display only low LD with the more common tagging SNPs, making GWAs underpowered to detect these associations [42]. Common causal variants, on the other hand, are more likely to be in strong LD with associated tagging SNPs and thus more suitable to be detected using GWA. However, the allelic frequency spectrum of common causal variants (minor allele frequency (MAF)≥ 5 %)

implies that these variants have only a minor effect on fitness and thus rather moderate to low effect sizes on the trait [41]. Strategies suggested to increase statistical power of GWAS include the collection of larger samples, the study of isolated or founder populations, or shifting focus from common (MAF≥ 5%) to rare single nucleotide polymorphisms (SNPs) (MAF< 5%) and other genetic variants, which might have a larger effect size on the trait [41].Particularly, large sample sizes will be required to determine genetic associations with even modest effects on the trait. Good examples of this have been demonstrated for common traits like height [43] and body mass index [44]. The number of significant genome-wide associations in GWAS of these traits were shown to be proportional to the sample size studied [42] with significant associations seen to double by using twice the sample size.

GWAS meta-analysis

To increase the sample size and therefore statistical power to identify association with common genetic variants, a meta-analysis of GWAS of hearing function from 8 samples was conducted. This analysis was performed in collaboration with the G-EAR consortium, which collected hearing and genotyping data originating from 7 isolated populations. The majority of GWAS analyses used in this GWAs meta-analyses were conducted by the G-EAR consortium previous to this collaboration [37,45] and kindly provided for collaboration with TwinsUK. The eight samples included: one female twin sample of Northern European origin (TwinsUK), 6 samples collected from isolated populations of Southern European origin (from Italy and Croatia) [37,45] as well as one sample of isolated populations situated along the Silk Road [46]. The meta-analysis presented an association between hearing ability and genetic variation as measured in 4939 individuals of different ethnic origin and population structure. A single SNP in intron 6 of the salt inducible kinase 3 gene was found genome-wide significantly associated with PC2 ($p=3.7 \times 10^{-8}$). Often in GWAS, multiple neighbouring SNPs are identified significantly associated with a trait due to underlying linkage disequilibrium with the marker SNP. The single SNP association determined for SNP rs681524 did not show this pattern. Thus the veracity of the finding was questionable. It may have been a single false positive representing a systematic error in genotyping or, more likely, imputation, across the study samples. SNP rs681524 was genotyped in the TwinsUK sample and imputed in all other samples included in the GWAS meta-analysis. Imputation accuracy determined as genotype concordance in two subsamples from Friuli Venezia Giulia and Carlantino was high (0.94-0.97). In addition, genotyping accuracy determined in TwinsUK reached a genotype concordance of 0.99. These findings contradicted false positive association due to an underlying systematic error. It

was further hypothesised that the lack of SNPs in LD with rs681524 might likely be due to an exceptional LD structure in this region. Examination of LD at the respective locus in three genetic panels (HapMap version 2 CEU panel using LocusZoom [47] , 1000 Genomes [48] and the UK10K TwinsUK whole genome sequencing sample) reported exceptionally low correlation of rs681524 with surrounding SNPs, supporting this hypothesis. This genetic architecture observed would usually be expected for rare genetic variants commonly defined as having a minor allele frequency of < 5% in the measured populations. Despite an overall relatively low frequency (≤ 8%) of the minor C allele at rs681524 in all 8 populations, it could not be considered rare.

The forest plot at rs681524 confirmed a consistent direction of effect for all included samples, which was significant (p≤0.05) in 3 of the 7 samples (TwinsUK, Friuli Venezia Guilia and Split). Heterogeneity between study samples could not account for this association (I=0.0, p=0.481). Thus there is some evidence that the association between hearing PC2 and rs681524 is likely a real one, which was reflected in all included study samples. The high number of genetic loci tested for association with phenotypes in GWAS today, increases the likelihood of false positive significant association by chance. Replication of association with a genetic variant in an independent sample is therefore considered standard to support true positive association. To decrease the likelihood of false positive association, stringent significance thresholds are applied ($p \leq 5 \times 10^{-8}$) based on Bonferroni corrections for multiple testing. However, these stringent thresholds will also exclude possible true positive associations of lower significance. It has even been suggested that the inclusion of less significant true positive associations might account for the missing heritability [49]. A recent study in the ARHI cohort reports that all GWAS SNPs collectively accounted for 22% of the phenotypic variance in ARHI [26].

Biological plausibility of salt inducible kinase 3

Due to the significant association between PC2 and a marker SNP in SIK3, it was decided to further investigate the biological plausibility of this gene. Salt inducible kinases belong to the family of cAMP activated serine threonine kinases [50]. Three family members have been described so far [50], and isoforms 1 and 2 have been the main focus of research. Sik1 has been shown to be expressed in the inner ear, in the sensory epithelium of the vestibular system, where it is thought to be involved in the formation of endolymphatic hydrops via interaction with phosphodiesterases [51]. Both Sik1 and Sik3 control histone deacetylases via phosphorylation and nuclear export [52,53,54]. The majority of *Sik3* knockout mice (*Sik3*$^{-/-}$) die at birth. Surviving pups present with skeletal abnormalities, reduced bodyweight and dwarfism. However,

abnormalities in the hearing ability of (*Sik3*$^{-/-}$) mice have not been reported yet. *Sik3* is assumed to regulate cholesterol bile acid homeostasis and lipid storage size and is essential for chondrocyte hypertrophy [53,55] and has recently been shown involved in the formation of regulatory macrophages [56].

Due to its significant genome-wide association with PC2 and the involvement of other salt-inducible kinases (SIKs) in the inner ear [51], the *SIK3* gene was considered a good candidate gene. Accordingly, it was decided to investigate the expression of *Sik3* in the inner ear of mouse models at different stages of development.

Immunohistochemistry in mouse models

Immunohistochemistry of Sik3 in the mouse inner ear showed expression of the gene in various structures of the inner essential for hearing ability including Inner and outer hair cells, a subgroup of glial cells in the spiral ganglion and resident macrophages in the stria vascularis. Expression was examined at three developmental stages, the day of birth (P0), 5 days postnatal (P5) and at 4 weeks of age (4w). While Sik3 was only expressed in hair cells during early development (P0 and P5), expression remained present up to 4 weeks of age in the spiral ganglion, stria vascularis and Reissner´s membrane. This might indicate differential function of this protein in different cells of the inner ear and different points in development. Whereas sik3 might serve a maintenance function in cells of the spiral ganglion, the expression profile reported here suggests a developmental function of this gene in hair cells. Although tempting to suggest a putative mechanistic link between Sik3 expression and hearing function throughout life, these suggestions should be tentative until more information can be gained from *sik3* knockout mice. In conclusion, we demonstrated the first genome-wide significant association between salt inducible kinase 3 and hearing function, with the expression profile of this gene in mice cochlea indicating a putative important function in hearing.

Further GWAS meta-analysis results

Despite the large overlap in samples between the original GWAS meta-analysis of hearing function in the G-EAR consortium conducted by *Girotto et al* [37] and the meta-analysis presented here of the G-EAR samples plus TwinsUK, only few associations were replicated in the current study. One of these was the association between PC1 and a SNP in the vicinity of metabotropic glutamate receptor type 8 (rs2687481, $p=1.07x10^{-7}$). Glutamate presents a major excitatory neurotransmitter in the central nervous system and the inner ear. A previous GWAS on ARHI found GRM7, another

close member of this family of glutamate receptors, strongly associated with hearing [6]. Although we could not replicate this exact association with GRM7, the association of two members of the glutamate receptors in independent studies of hearing function and ARHI supports an essential function of these receptors in hearing.

The low overlap of top associated SNPs between the original GWAS meta-analysis [37] and the meta-analysis presented here might be indicative of multiple genetic variants contributing to hearing function, each with only moderate effect on the trait. Replication of these associations in an independent sample is considered the gold standard validation of true positive associations, but difficult to achieve with low to moderate sample sizes. Populations included in the original meta-analysis were ethically more homogenous [37], being collected from Southern European (from Italy and Croatia) isolated populations, compared to the current meta-analysis, which included an additional sample of Northern European origin and a selection of isolated populations situated along the Silk Road.

Epigenetic Analysis

Many age-related common traits show moderate to high heritability estimates. However, despite a few exceptions like age-related macular degeneration [57], genetic variants genome-wide significantly associated with age-related traits as identified in GWAS currently explain only a minority of phenotypic variation [41]. While the genetic make up of an individual is determined at conception, epigenetic modifications can occur throughout life and have been shown associated with environmental exposure [58,59]. Epigenetic marks have been identified as the cause of various disorders (i.e. various forms of cancers [60], Alzheimer's disease[61,62] and Rett syndrome [63]) and have been shown to change throughout life associated with various environmental factors [59]. Previous studies have shown that epigenetic differences within monozygotic twin pairs may explain phenotypic discordance [64] with MZ twin siblings leading discordant lifestyles showing the strongest epigenetic differences [59].Furthermore, epigenetic modifications as a cause of disease are particular interesting due to their disease mechanisms and might help identify novel targets for treatment due to their potential reversibility [65].

Epigenome-wide association study

In the epigenetic analysis of hearing ability, two study designs were applied: an epigenome-wide association study (EWAS) and a MZ co-twin analysis to take advantage of MZ twins having identical DNA code and thus adjust for any differences

DNA code may make on epigenetic marks. Firstly, all samples with available pure-tone audiometry and DNA methylation measured using the Illumina HumanMethylation27K array [66] (n=115) were used in an EWAS in which, analogous to GWAS association with ~ 24,000 epigenetic marks were examined across all autosomal chromosomes. Despite the relatively small sample size available for the EWAS, one CpG probe (cg01161216) in the promoter region of the transcription factor 25 (*TCF25*) gene was associated reaching suggestive epigenome-wide significance (considering a Bonferroni correction for 14,495 genes) in the discovery EWAS. This finding was replicated in females from the TwinsUK registry (n=203) unrelated to the discovery samples with DNA methylation measured using the Illumina HumanMethylation 450k array [67]. An additional technical replication was performed using an alternative method [68] to quantify DNA methylation (n=46). The association was not explained by an underlying association with age, underlying genome-wide association with genetic variants or blood cell heterogeneity in the blood samples used to measure DNA methylation.

Methylation levels at the *TCF25* promoter were reduced in samples with low hearing ability. The effect of DNA methylation at *TCF25* on the expression of this gene was determined using the available expression data available for the same twins previously used in the MuTHER study [69]. This sample comprised 172 individuals. DNA methylation at *TCF25* was negatively correlated with gene expression (r=-0.02), a finding, which is in accordance with the hypothesis that DNA methylation at gene promoters represses gene expression.

TCF25, also referred to as human homologue of *NULP1*, belongs to the family of helix-loop-helix transcription factors and encodes a protein with a novel transcriptional repressive domain, which can repress the transcription of other genes, particular the serum response factor [70]. This gene has been further shown to be expressed in mouse dorsal root ganglia during embryonic development [71]. The serum response factor is involved in cell-cycle regulation, apoptosis and cell differentiation and might thereby affect ageing processes in the inner ear. Due to the wide expression and transcriptional regulation of TCF25 on other genes, suggesting a clear mechanistic link is problematic. Further studies in model organisms (i.e. mice or rats) investigating changes in DNA methylation at *TCF25* throughout development and ageing and their effect on hearing function will be required to shed light on putative mechanistic links.

Monozygotic twin discordance study

All MZ twin pairs (n=21 pairs) selected from the EWAS discovery sample were selected for an additional co-twin discordance study, where trait discordance within twin pairs was compared to inter-twin pair DNA methylation differences at CpG sites epigenome-wide. MZ twin siblings offer a particular advantage in the second study design as they are perfectly matched for age, gender and genetic variation. In addition, most twin pairs show a high proportion of shared environmental exposure compared to normal siblings or unrelated individuals. Although discordance in hearing ability was not great within our sample, strong associations were observed ($p \geq 2.5 \times 10^{-5}$). One of the most strongly correlated probes for PC1 discordance was found in the promoter of the myocyte enhance factor 2 D (*MEF2D*) gene. The myocyte enhancer factor family of transcription factors is involved in neuronal development and differentiation under regulation of class 2 histone deacetylases. The associated gene (*MEF2D*) is expressed in mouse cochlear neurons and sensory cells at P15 and was diminished in IGF knockout mice showing sensorineural hearing loss [72]. Reduced expression of *MEF2D* due to DNA methylation could result in sensorineural hearing loss in humans, as reported for mouse models [72]. Discordance for PC2 was most strongly correlated with differential methylation at *CCNDBP1*, which represses transcriptional activity of cyclin D1 and has been shown to directly interact with Sirt6 [73], a class 3 histone deacetylase linked to cancer and ageing [74]. There were limitations to the MZ discordance analysis, like the limited discordance in hearing ability within the available twin siblings. In addition, we lacked subjects to replicate our findings in an independent twin sample. Future studies should thus focus on the collection of particular hearing discordant elderly twin siblings, to determine whether epigenetic differences could account for their phenotypic discordance. A manuscript describing the epigenetic analyses has been submitted for publication.

Conclusion

The findings presented here and in previous studies show that ARHI is dependent both on environmental exposure and genotype. This PhD thesis did not only generate an excellent new cohort to study age-related hearing impairment in the future, but also obtain the first genome-wide significant association with hearing function and demonstrate associations between hearing ability with age and epigenetic modifications.

Despite moderate heritability estimates determined for ARHI in this and previous studies, genome-wide significant associations in GWAS analysis of ARHI could not be

obtained due to limited sample sizes of separate analysis previously. We were able to show by combining our data in collaboration with other researchers that variation in hearing function can be explained by association with common genetic variants. However, even larger collaborations will be essential to achieve sufficient sample sizes required. The association of *SIK3* with hearing function had not been predicted previously, supporting the use of GWAS analysis to determine novel genes involved in this common complex trait. Immunohistochemistry of sik3 in mouse cochlea supports an important function of this gene in development and maintenance of various cochlear structures, including inner and outer hair cells.

Furthermore, we were the first to demonstrate an association between DNA methylation in the promoter of TCF25 and hearing ability with age. This result was particularly exciting considering the limited sample size, indicating that these modifications might have a strong enough effect on hearing ability with age to be determined even in moderately sized samples. We hope that these findings will motivate other researchers to further investigate the possible effects of epigenetic modifications of hearing ability with age and thereby open new doors to better understand this common trait.

Future studies

Future studies of ARHI should ideally account for three factors (genotype, epigenetic modifications and environmental exposure) and interactions of the same. Combining and expanding existing datasets will be essential to reach the large sample sizes required for these complex studies.

Environmental factors

According to our heritability estimates, 66-80% of variance in speech perception in noise was determined by environmental exposure not shared within twin pairs. It would there for be interesting to investigate associations with environmental exposure and speech perception in future studies.

Genetic factors

Current studies have mainly focused on common genetic variants in ARHI and hearing function. We were able to show that genome-wide significant associations can be determined with sufficiently large sample sizes. However, even larger sample sizes might be required to explain the moderate to high heritability of this common complex trait. In addition, the increased availability of whole genome sequencing data in hearing cohorts will facilitate investigations into the association with rare variants.

Epigenetic factors

To our knowledge, we were the first to demonstrate the association between cytosine DNA methylation and ARHI epigenome-wide and hope that our results will motivate other researchers to investigate epigenetic factors in ARHI. Future studies should focus on larger sample sizes, different epigenetic modifications (ie histone methylation and acetylation) and higher epigenome-wide coverage. Epigenetic changes have been shown associated with environmental exposure. In ARHI the association between epigenetic modifications and environmental risk factors like noise exposure could provide a link between environmental assault and changes in gene expression. Furthermore, future studies should build upon the MZ discordance analysis by collecting of specifically hearing discordant twin pairs. Follow up of the differentially methylated regions associated with hearing ability with age in model organisms could give further insight into the biological mechanisms of these genes. In addition, model organisms could be kept under equal environmental conditions, thereby controlling for environmental exposure, which is currently impossible in humans.

References

1. AOHL (2011) Hearing matters. London: Action on Hearing Loss.
2. Dalton DS, Cruickshanks KJ, Klein BE, Klein R, Wiley TL, et al. (2003) The impact of hearing loss on quality of life in older adults. Gerontologist 43: 661-668.
3. Schuknecht HF, Gacek MR (1993) Cochlear pathology in presbycusis. Ann Otol Rhinol Laryngol 102: 1-16.
4. Fransen E, Topsakal V, Hendrickx JJ, Van Laer L, Huyghe JR, et al. (2008) Occupational noise, smoking, and a high body mass index are risk factors for age-related hearing impairment and moderate alcohol consumption is protective: a European population-based multicenter study. JARO-Journal of the Association for Research in Otolaryngology 9: 264-276.
5. Cruickshanks KJ, Wiley TL, Tweed TS, Klein BEK, Klein R, et al. (1998) Prevalence of Hearing Loss in Older Adults in Beaver Dam, Wisconsin: The Epidemiology of Hearing Loss Study. American Journal of Epidemiology 148: 879-886.
6. Friedman RA, Van Laer L, Huentelman MJ, Sheth SS, Van Eyken E, et al. (2009) GRM7 variants confer susceptibility to age-related hearing impairment. Hum Mol Genet 18: 785-796.

7. Garringer HJ, Pankratz ND, Nichols WC, Reed T (2006) Hearing impairment susceptibility in elderly men and the DFNA18 locus. Arch Otolaryngol Head Neck Surg 132: 506-510.

8. Gates GA, Couropmitree NN, Myers RH (1999) Genetic associations in age-related hearing thresholds. Arch Otolaryngol Head Neck Surg 125: 654-659.

9. BSA (2011) Recommended Procedure for Pure-tone air-conduction and bone-conduction threshold audiometry with and without masking. Reading: British Society of Audiology.

10. Unal M, Tamer L, Dogruer ZN, Yildirim H, Vayisoglu Y, et al. (2005) N-acetyltransferase 2 gene polymorphism and presbycusis. Laryngoscope 115: 2238-2241.

11. Cruickshanks KJ, Wiley TL, Tweed TS, Klein BE, Klein R, et al. (1998) Prevalence of hearing loss in older adults in Beaver Dam, Wisconsin. The Epidemiology of Hearing Loss Study. Am J Epidemiol 148: 879-886.

12. Christensen K, Wienke A, Skytthe A, Holm NV, Vaupel JW, et al. (2001) Cardiovascular mortality in twins and the fetal origins hypothesis. Twin Research 4: 344-349.

13. Andrew T, Hart DJ, Snieder H, de Lange M, Spector TD, et al. (2001) Are twins and singletons comparable? A study of disease-related and lifestyle characteristics in adult women. Twin Res 4: 464-477.

14. Boomsma D, Busjahn A, Peltonen L (2002) Classical twin studies and beyond. Nat Rev Genet 3: 872-882.

15. Rijsdijk FV, Sham PC (2002) Analytic approaches to twin data using structural equation models. Briefings in Bioinformatics 3: 119-133.

16. World Health Organisation (2011) Grades of hearing impairment. In: Organization WH, editor. Prevention of Blindness and Deafness Geneva: World Health Organization.

17. Viljanen A, Era P, Kaprio J, Pyykkö I, Koskenvuo M, et al. (2007) Genetic and Environmental Influences on Hearing in Older Women. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences 62: 447-452.

18. Huyghe JR, Van Laer L, Hendrickx JJ, Fransen E, Demeester K, et al. (2008) Genome-wide SNP-based linkage scan identifies a locus on 8q24 for an age-related hearing impairment trait. Am J Hum Genet 83: 401-407.

19. Fransen E, Van Laer L, Lemkens N, Caethoven G, Flothmann K, et al. (2004) A novel Z-score-based method to analyze candidate genes for age-related hearing impairment. Ear Hear 25: 133-141.

20. Allen PD, Eddins DA (2010) Presbycusis phenotypes form a heterogeneous continuum when ordered by degree and configuration of hearing loss. Hearing Research 264: 10-20.

21. Demeester K, Van Wieringen A, Hendrickx J-j, Topsakal V, Huyghe J, et al. (2010) Heritability of audiometric shape parameters and familial aggregation of presbycusis in an elderly Flemish population. Hearing Research 265: 1-10.

22. Demeester K, van Wieringen A, Hendrickx J-j, Topsakal V, Fransen E, et al. (2009) Audiometric shape and presbycusis. International Journal of Audiology 48: 222-232.

23. (2011) Genetic variants in novel pathways influence blood pressure and cardiovascular disease risk. Nature 478: 103-109.

24. Evangelou E, Valdes AM, Kerkhof HJM, Styrkarsdottir U, Zhu Y, et al. (2011) Meta-analysis of genome-wide association studies confirms a susceptibility locus for knee osteoarthritis on chromosome 7q22. Annals of the Rheumatic Diseases 70: 349-355.

25. Identification of new susceptibility loci for osteoarthritis (arcOGEN): a genome-wide association study. The Lancet 380: 815-823.

26. Fransen E, Bonneux S, Corneveaux JJ, Schrauwen I, Di Berardino F, et al. (2014) Genome-wide association analysis demonstrates the highly polygenic character of age-related hearing impairment. European Journal of Human Genetics.

27. Dalton DS, Cruickshanks KJ, Wiley TL, Klein BEK, Klein R, et al. (2001) Association of Leisure-Time Noise Exposure and Hearing Loss: Asociación entre exposición a ruido durante el tiempo libre e hipoacusia. International Journal of Audiology 40: 1-9.

28. Nash SD, Cruickshanks KJ, Klein R, Klein BEK, Nieto FJ, et al. (2011) The prevalence of hearing impairment and associated risk factors: the Beaver Dam Offspring Study. Archives of Otolaryngology—Head & Neck Surgery 137: 432.

29. Torre P, III, Cruickshanks KJ, Klein BEK, Klein R, Nondahl DM (2005) The Association Between Cardiovascular Disease and Cochlear Function in Older Adults. J Speech Lang Hear Res 48: 473-481.

30. Karlsson KK, Harris JR, Svartengren M (1997) Description and primary results from an audiometric study of male twins. Ear Hear 18: 114-120.

31. Schousboe K, Willemsen G, Kyvik KO, Mortensen J, Boomsma DI, et al. (2003) Sex Differences in Heritability of BMI: A Comparative Study of Results from Twin Studies in Eight Countries. Twin Research and Human Genetics 6: 409-421.

32. Deary I, Johnson W, Houlihan LM (2009) Genetic foundations of human intelligence. Human Genetics 126: 215-232.

33. Reynolds CA, Finkel D, McArdle JJ, Gatz M, Berg S, et al. (2005) Quantitative genetic analysis of latent growth curve models of cognitive abilities in adulthood. Developmental psychology 41: 3.

34. McGue M, Christensen K (2002) The heritability of level and rate-of-change in cognitive functioning in Danish twins aged 70 years and older. Experimental aging research 28: 435-451.

35. Herskind AM, McGue M, Holm NV, Sörensen TI, Harvald B, et al. (1996) The heritability of human longevity: a population-based study of 2872 Danish twin pairs born 1870–1900. Human Genetics 97: 319-323.

36. Van Laer L, Huyghe JR, Hannula S, Van Eyken E, Stephan DA, et al. (2010) A genome-wide association study for age-related hearing impairment in the Saami. Eur J Hum Genet 18: 685-693.

37. Girotto G, Pirastu N, Sorice R, Biino G, Campbell H, et al. (2011) Hearing function and thresholds: a genome-wide association study in European isolated populations identifies new loci and pathways. Journal of Medical Genetics 48: 369-374.

38. Lutman ME, Hall SJ, Athalye S (2006) Development of a telephone hearing test.

39. Smits C, Kapteyn TS, Houtgast T (2004) Development and validation of an automatic speech-in-noise screening test by telephone. International Journal of Audiology 43: 15-28.

40. Smits C, Merkus P, Houtgast T (2006) How we do it: The Dutch functional hearing–screening tests by telephone and internet. Clinical Otolaryngology 31: 436-440.

41. Manolio TA, Collins FS, Cox NJ, Goldstein DB, Hindorff LA, et al. (2009) Finding the missing heritability of complex diseases. Nature 461: 747-753.

42. Visscher Peter M, Brown Matthew A, McCarthy Mark I, Yang J (2012) Five Years of GWAS Discovery. The American Journal of Human Genetics 90: 7-24.

43. Visscher PM (2008) Sizing up human height variation. Nature Genetics 40: 489-490.

44. Speliotes EK, Willer CJ, Berndt SI, Monda KL, Thorleifsson G, et al. (2010) Association analyses of 249,796 individuals reveal 18 new loci associated with body mass index. Nature Genetics 42: 937-948.

45. Bedin E, Franzè A, Zadro C, Persico MG, Ciullo M, et al. (2009) Age-related hearing loss in four Italian genetic isolates: an epidemiological study. International Journal of Audiology 48: 465-472.

46. Girotto G, Pirastu N, Gasparini A, D'Adamo P, Gasparini P (2011) Frequency of hearing loss in a series of rural communities of five developing countries located along the Silk Road. Audiological Medicine 9: 135-140.

47. Pruim RJ, Welch RP, Sanna S, Teslovich TM, Chines PS, et al. (2010) LocusZoom: regional visualization of genome-wide association scan results. Bioinformatics 26: 2336-2337.

48. (2012) An integrated map of genetic variation from 1,092 human genomes. Nature 491: 56-65.

49. Lee Sang H, Wray Naomi R, Goddard Michael E, Visscher Peter M (2011) Estimating Missing Heritability for Disease from Genome-wide Association Studies. The American Journal of Human Genetics 88: 294-305.

50. Katoh Y, Takemori H, Horike N, Doi J, Muraoka M, et al. (2004) Salt-inducible kinase (SIK) isoforms: their involvement in steroidogenesis and adipogenesis. Molecular and Cellular Endocrinology 217: 109-112.

51. Degerman E, Rauch U, Göransson O, Lindberg S, Hultgårdh A, et al. (2011) Identification of new signaling components in the sensory epithelium of human saccule. Frontiers in neurology 2: 48.

52. Berdeaux R, Goebel N, Banaszynski L, Takemori H, Wandless T, et al. (2007) SIK1 is a class II HDAC kinase that promotes survival of skeletal myocytes. Nat Med 13: 597-603.

53. Sasagawa S, Takemori H, Uebi T, Ikegami D, Hiramatsu K, et al. (2012) SIK3 is essential for chondrocyte hypertrophy during skeletal development in mice. Development 139: 1153-1163.

54. Walkinshaw DR, Weist R, Kim G-W, You L, Xiao L, et al. (2013) The tumor suppressor kinase LKB1 activates SIK2 and SIK3 to stimulate nuclear export of class IIa histone deacetylases. Journal of Biological Chemistry.

55. Uebi T, Itoh Y, Hatano O, Kumagai A, Sanosaka M, et al. (2012) Involvement of SIK3 in Glucose and Lipid Homeostasis in Mice. PLoS One 7: e37803.

56. Clark K, MacKenzie KF, Petkevicius K, Kristariyanto Y, Zhang J, et al. (2012) Phosphorylation of CRTC3 by the salt-inducible kinases controls the interconversion of classically activated and regulatory macrophages. Proceedings of the National Academy of Sciences 109: 16986-16991.

57. Gibbs D, Yang Z, Constantine R, Ma X, Camp NJ, et al. (2008) Further mapping of 10q26 supports strong association of< i> HTRA1</i> polymorphisms with age-related macular degeneration. Vision research 48: 685-689.

58. Wong CCY, Caspi A, Williams B, Craig IW, Houts R, et al. (2010) A longitudinal study of epigenetic variation in twins. Epigenetics 5: 516-526.

59. Fraga MF, Ballestar E, Paz MF, Ropero S, Setien F, et al. (2005) Epigenetic differences arise during the lifetime of monozygotic twins. Proceedings of the National Academy of Sciences of the United States of America 102: 10604-10609.

60. Jones PA, Baylin SB (2002) The fundamental role of epigenetic events in cancer. Nature reviews genetics 3: 415-428.

61. Mastroeni D, McKee A, Grover A, Rogers J, Coleman PD (2009) Epigenetic Differences in Cortical Neurons from a Pair of Monozygotic Twins Discordant for Alzheimer's Disease. PLoS One 4: e6617.

62. Mastroeni D, Grover A, Delvaux E, Whiteside C, Coleman PD, et al. (2010) Epigenetic changes in Alzheimer's disease: Decrements in DNA methylation. Neurobiology of Aging 31: 2025-2037.

63. Shahbazian MD, Zoghbi HY (2002) Rett syndrome and MeCP2: linking epigenetics and neuronal function. The American Journal of Human Genetics 71: 1259-1272.

64. Poulsen P, Esteller M, Vaag A, Fraga MF (2007) The epigenetic basis of twin discordance in age-related diseases. Pediatric research 61: 38R-42R.

65. Egger G, Liang G, Aparicio A, Jones PA (2004) Epigenetics in human disease and prospects for epigenetic therapy. Nature 429: 457-463.

66. Bibikova M, Le J, Barnes B, Saedinia-Melnyk S, Zhou L, et al. (2009) Genome-wide DNA methylation profiling using Infinium® assay. Epigenomics 1: 177-200.

67. Bibikova M, Barnes B, Tsan C, Ho V, Klotzle B, et al. (2011) High density DNA methylation array with single CpG site resolution. Genomics 98: 288-295.

68. Down TA, Rakyan VK, Turner DJ, Flicek P, Li H, et al. (2008) A Bayesian deconvolution strategy for immunoprecipitation-based DNA methylome analysis. Nat Biotech 26: 779-785.

69. Grundberg E, Small KS, Hedman AK, Nica AC, Buil A, et al. (2012) Mapping cis- and trans-regulatory effects across multiple tissues in twins. Nat Genet 44: 1084-1089.

70. Cai Z, Wang Y, Yu W, Xiao J, Li Y, et al. (2006) hnulp1, a basic helix-loop-helix protein with a novel transcriptional repressive domain, inhibits transcriptional activity of serum response factor. Biochemical and Biophysical Research Communications 343: 973-981.

71. Olsson M, Durbeej M, Ekblom P, Hjalt T (2002) Nulp1, a novel basic helix-loop-helix protein expressed broadly during early embryonic organogenesis and prominently in developing dorsal root ganglia. Cell and Tissue Research 308: 361-370.

72. Sanchez-Calderon H, Rodriguez-de La Rosa L, Milo M, Pichel JG, Holley M, et al. (2010) RNA microarray analysis in prenatal mouse cochlea reveals novel IGF-I target genes: implication of MEF2 and FOXM1 transcription factors. PLoS One 5: e8699.

73. Ma W, Stafford LJ, Li D, Luo J, Li X, et al. (2007) GCIP/CCNDBP1, a helix–loop–helix protein, suppresses tumorigenesis. Journal of cellular biochemistry 100: 1376-1386.

74. Denu JM, Gottesfeld JM (2012) Minireview Series on Sirtuins: From Biochemistry to Health and Disease. Journal of Biological Chemistry 287: 42417-42418.

Appendix chapter 2

1. Copy of hearing questionnaire

Learning, memory and hearing questionnaire

Healthy Ageing Twin Study

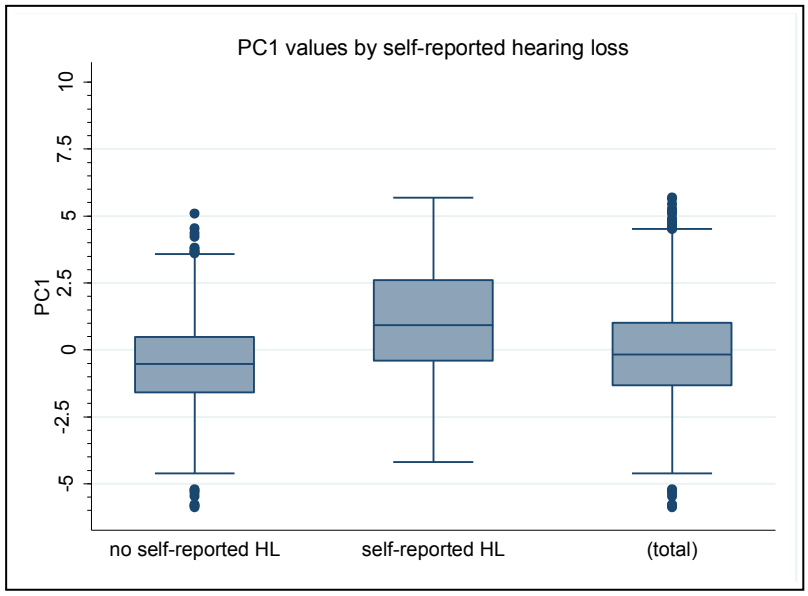
Your hearing:

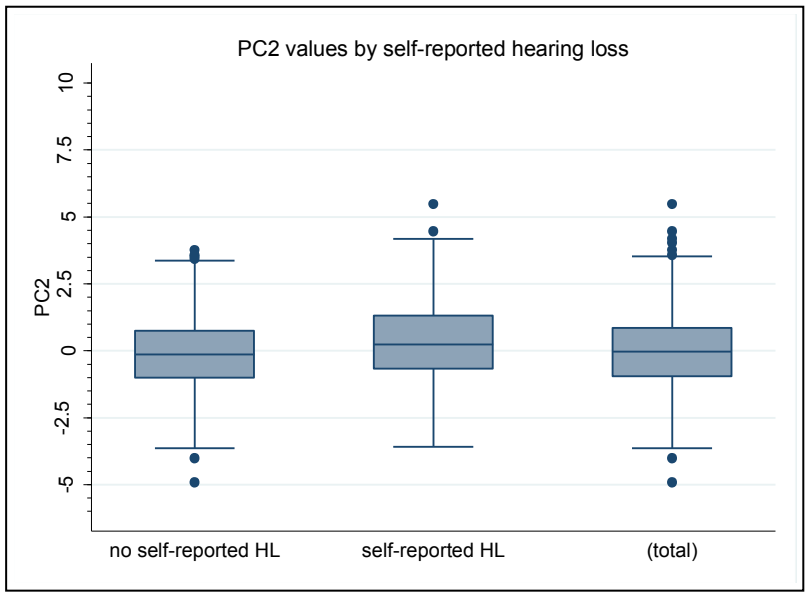
2.1 **Do you have any difficulty with your hearing?**	☐ No	☐ Yes	☐ Not known
2.2 **Have you ever had an ear disease?** (> more than)	☐ No	☐ **Yes** Acute ear inflammation **in childhood** (>3 times) with pain, discharge.	☐ Not known
		☐ **Yes** Acute ear inflammation **as an adult** (>3 times) with pain and discharge	☐ Not known
		☐ **Yes** Chronic ear inflammation with a feeling of deafened ear, discharge from ear (lasting >3 months)	☐ Not known
2.3 **Or an ear operation?**	☐ No	☐ **Yes** Plastic tube through eardrum ☐ R ☐ L	☐ Not known
		☐ **Yes** Other eardrum operation ☐ R ☐ L	☐ Not known
		☐ **Yes** Ossicle operation ☐ R ☐ L	☐ Not known
		☐ **Yes** Infection in bones behind ear (Mastoiditis) ☐ R ☐ L	☐ Not known
		☐ **Yes** Cholesteatoma of middle ear ☐ R ☐ L	☐ Not known
		☐ **Yes** Otosclerosis (broken ossicle) ☐ R ☐ L	☐ Not known
2.4 **Have you ever experienced an explosion or gunfire** which caused immediate HL or tinnitus?	☐ No	☐ Yes	☐ Not known
2.5 **Have you been frequently exposed to loud noise in your leisure time without using protection.** (Frequently would be more than once a month over several years)			
	☐ No	☐ Yes – loud music ☐ Yes – noisy handiwork / power tools ☐ Yes – gunshots	☐ Not known
2.6 **Do you wear a hearing aid?**	☐ No	☐ Yes	☐ Not known

3.1 **What best describes your main occupation throughout most of your life?** ☐ Professional or managerial ☐ Non-manual or clerical ☐ Manual ☐ Housewife ☐ Student ☐ None	3.2 **Have you <u>ever</u> worked in a place that was so noisy you had to shout to be heard**? ☐ No, never ☐ Yes, for less than 1 year ☐ Yes, for 1-5 years ☐ Yes, for more than 5 years

2. Self reported HL

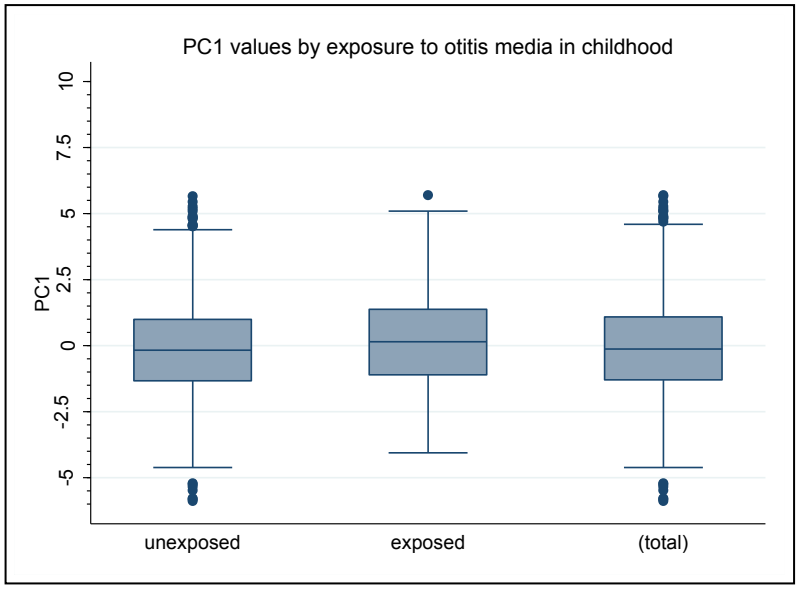
	age-adjusted PC1 (mean±SD)	age-adjusted PC2 (mean±SD)	n
no self-reported HL	-0.5141 ±1.6570	-0.1141 ±1.2845	881
self-reported HL	1.0515 ±2.0165	0.3003 ±1.4790	347
total	-0.0717 ±1.9008	0.0030 ±1.3546	1228
Student's t-test	p<0.000	p<0.000	-

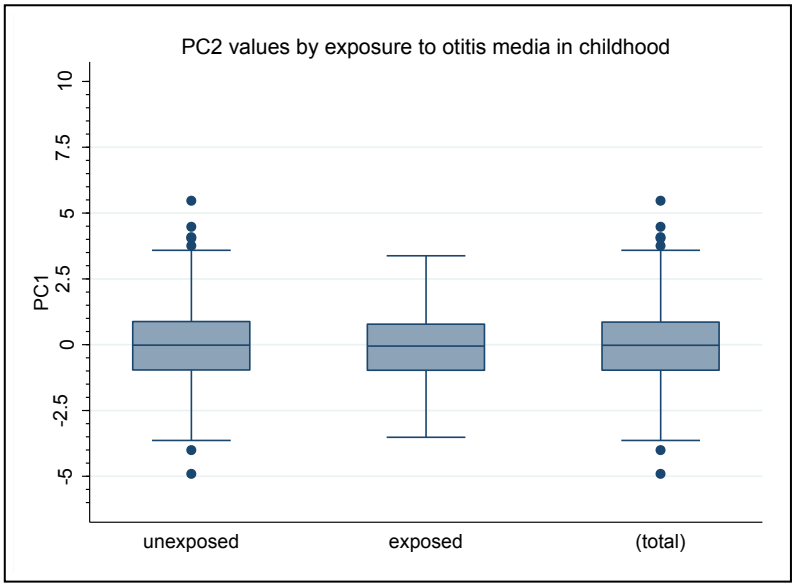




3. Exposure to otitis media during childhood

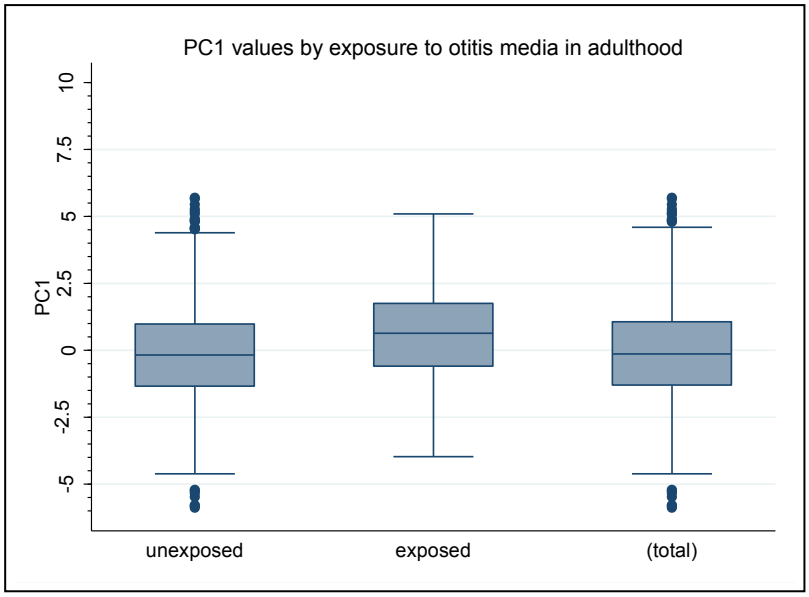
	age-adjusted PC1 (mean±SD)	age-adjusted PC2 (mean±SD)	n
unexposed	-0.0689 ±1.8854	0.0082 ±1.3628	996
exposed	0.2848 ±2.0200	-0.0885 ±1.3166	127
total	-0.0289 ±1.9034	-0.0027 ±1.3574	1123
Student's t-test	p=0.0631	p=0.4385	-

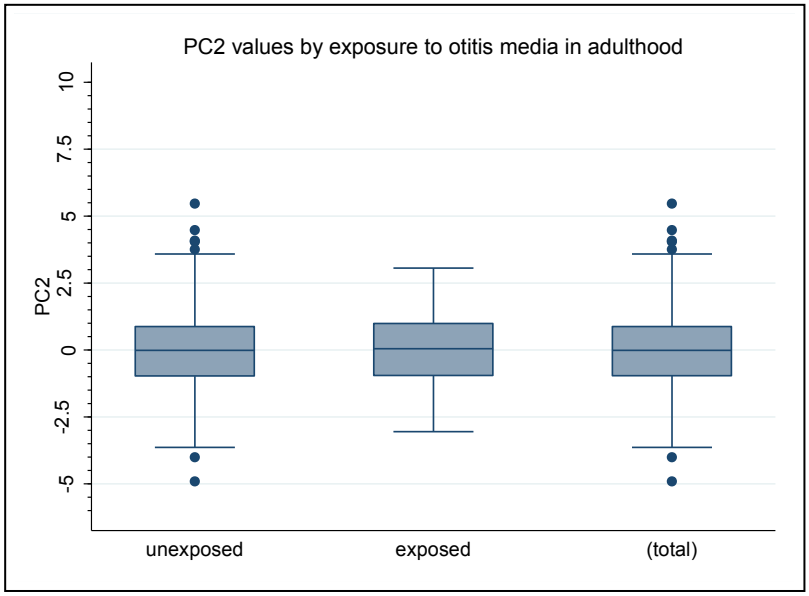




4. Exposure to otitis media in adulthood

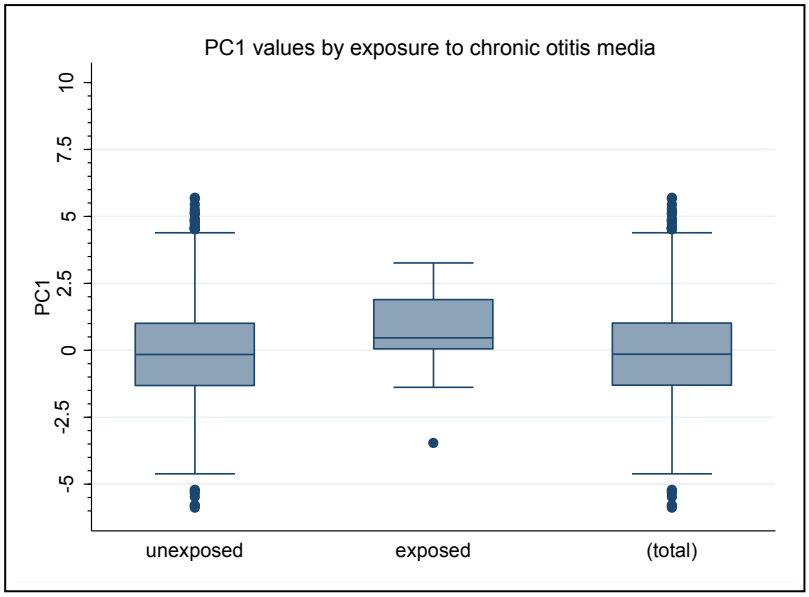
	age-adjusted PC1 (mean±SD)	age-adjusted PC2 (mean±SD)	n
unexposed	-0.0773 ±1.8848	0.0060 ±1.3616	1075
exposed	0.6497 ±1.8545	0.0642 ±1.3794	61
total	-0.0383 ±1.8895	0.0091 ±1.3620	1136
Student's t-test	p=0.0041	p=0.7493	-

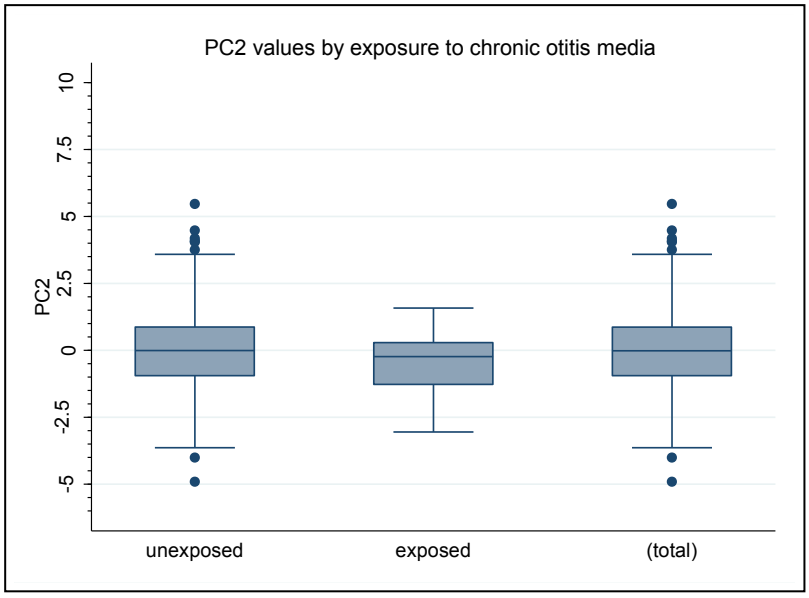




5. Exposure to chronic otitis media

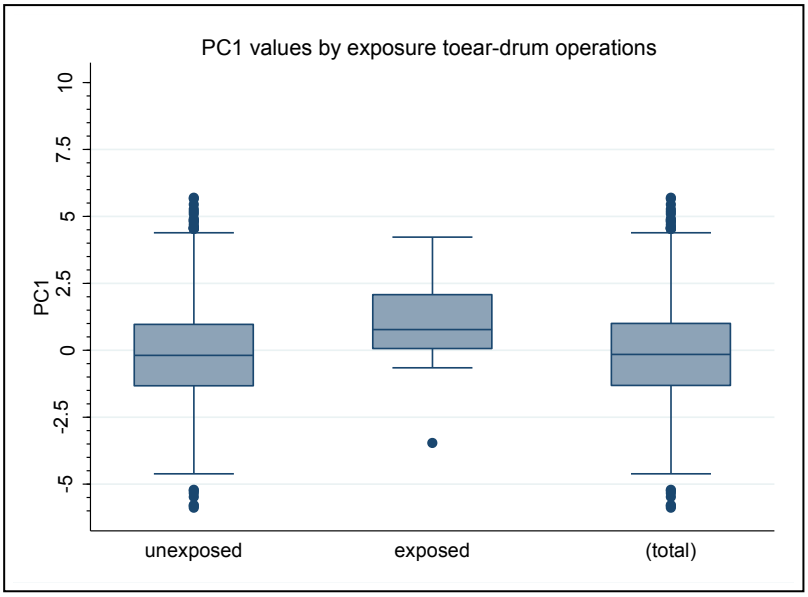
	age-adjusted PC1 (mean±SD)	age-adjusted PC2 (mean±SD)	n
unexposed	-0.0690 ±1.8795	0.0101 ±1.3574	1268
exposed	0.7448 ±1.7267	-0.4087 ±1.2647	20
total	-0.0564 ±1.8793	0.0036 ±1.3566	1288
Student's t-test	p=0.0499	p=0.1580	-

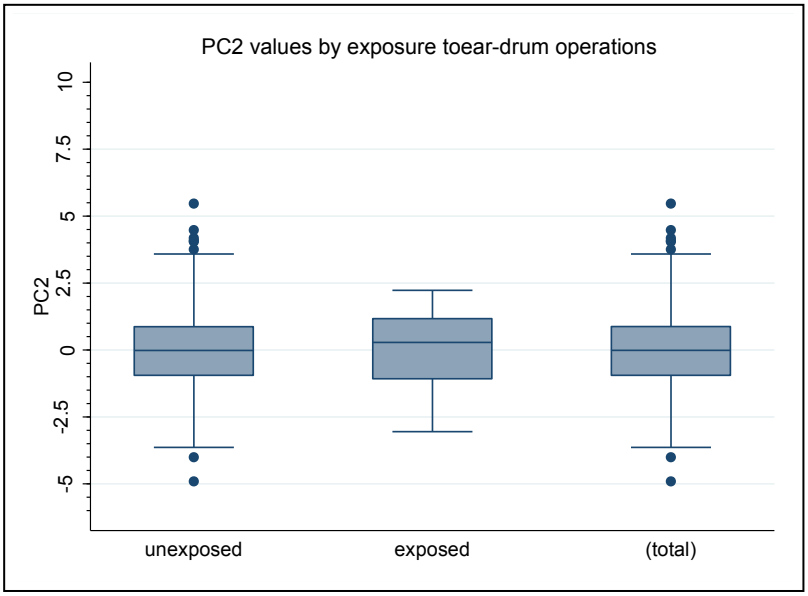




6. Exposure to eardrum operations

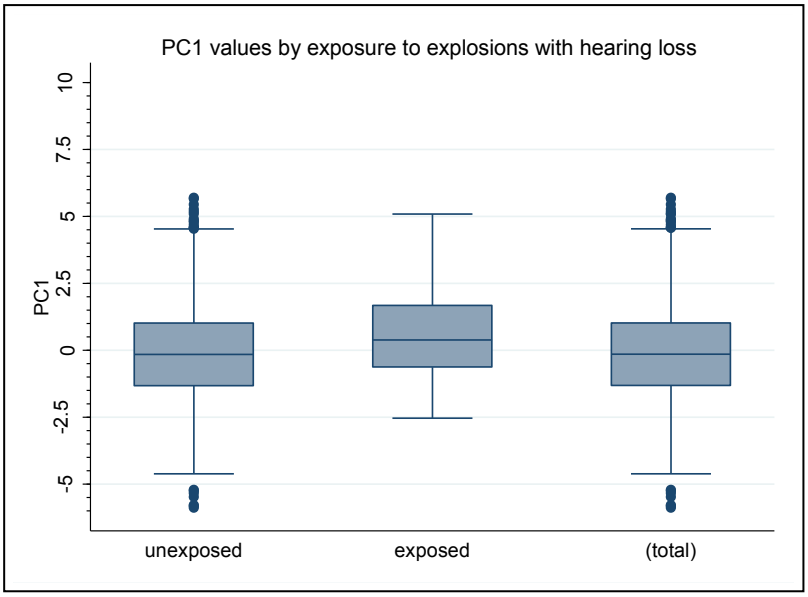
	age-adjusted PC1 (mean±SD)	age-adjusted PC2 (mean±SD)	n
unexposed	-0.0965 ±1.8647	0.0109 ±1.3541	1268
exposed	1.0371 ±1.6495	-0.0267 ±1.5196	26
total	-0.0738 ±1.8669	0.0101 ±1.3569	1294
Student's t-test	p=0.0019	p=0.9015	-

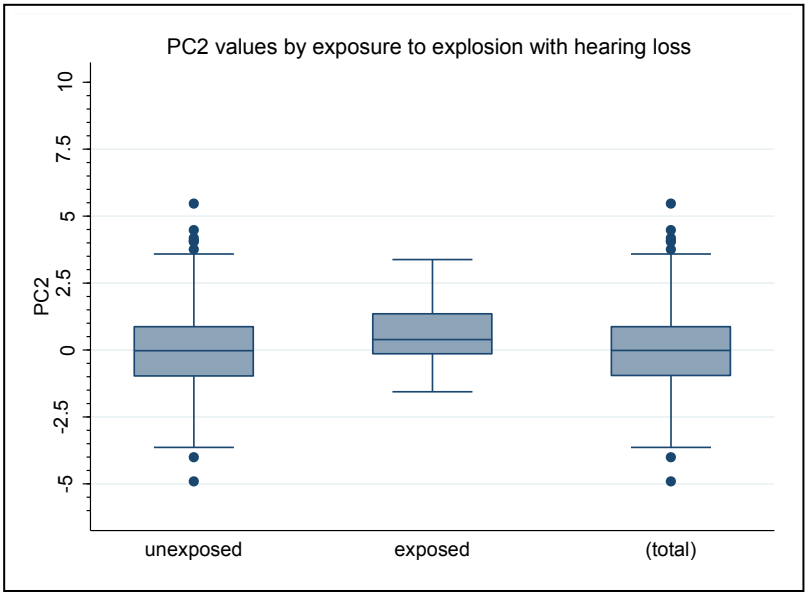




7. Exposure to explosions with subsequent sudden HL

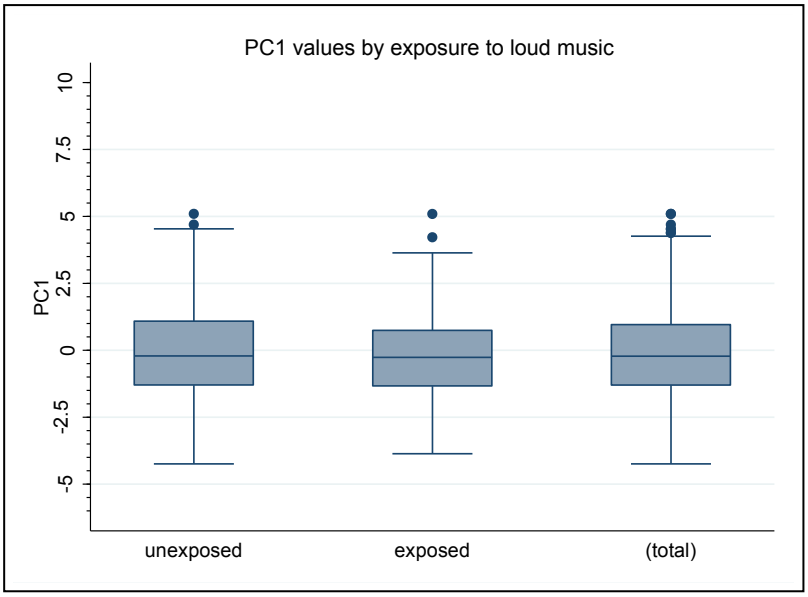
	age-adjusted PC1 (mean±SD)	age-adjusted PC2 (mean±SD)	n
unexposed	-0.0706 ±1.8777	-0.0115 ±1.3559	1267
exposed	0.6238 ±2.0718	0.5927 ±1.2808	28
total	-0.0556 ±1.8839	0.0016 ±1.3567	1295
Student's t-test	p=0.0897	p=0.0200	-

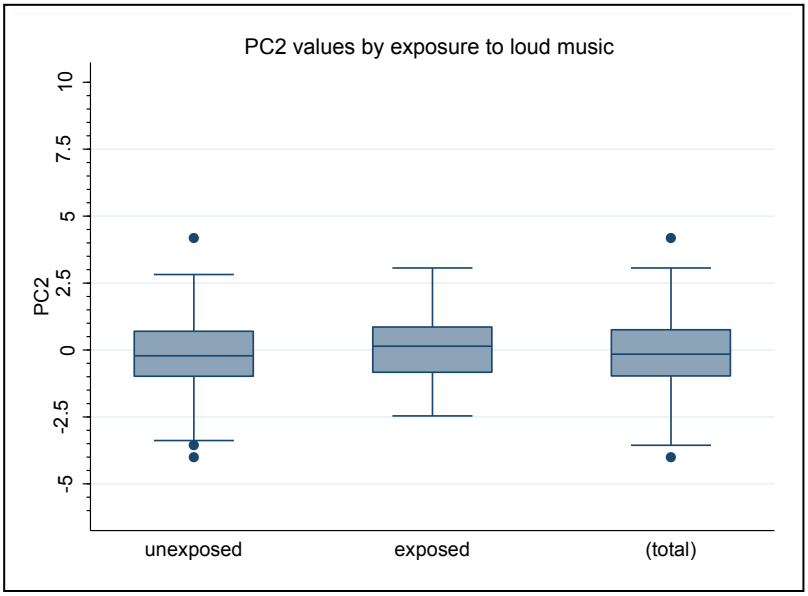




8. Exposure to loud music

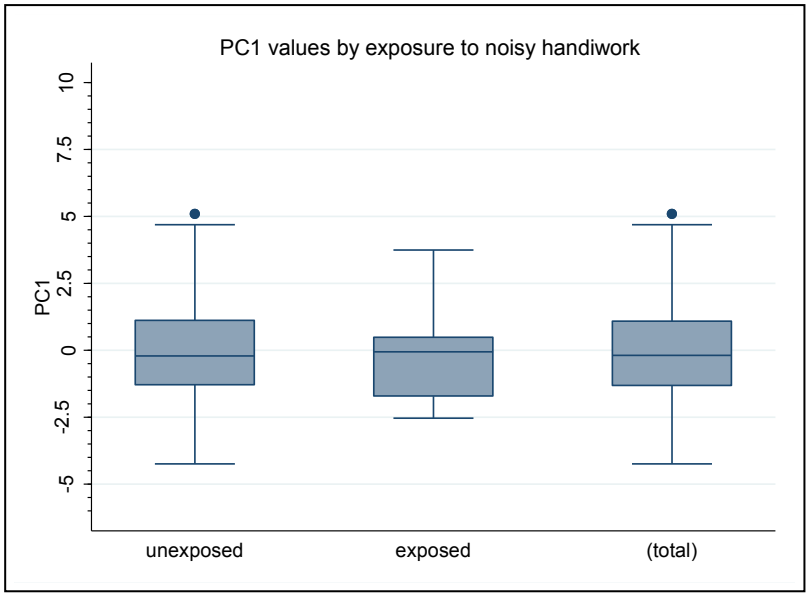
	age-adjusted PC1 (mean±SD)	age-adjusted PC2 (mean±SD)	n
unexposed	-0.0362 ±1.8461	-0.1709 ±1.3235	336
exposed	-0.1590 ±1.7730	0.1031 ±1.3274	92
total	-0.0626 ±1.8293	-0.1120 ±1.3276	428
Student's t-test	p=0.5606	p=0.0814	-

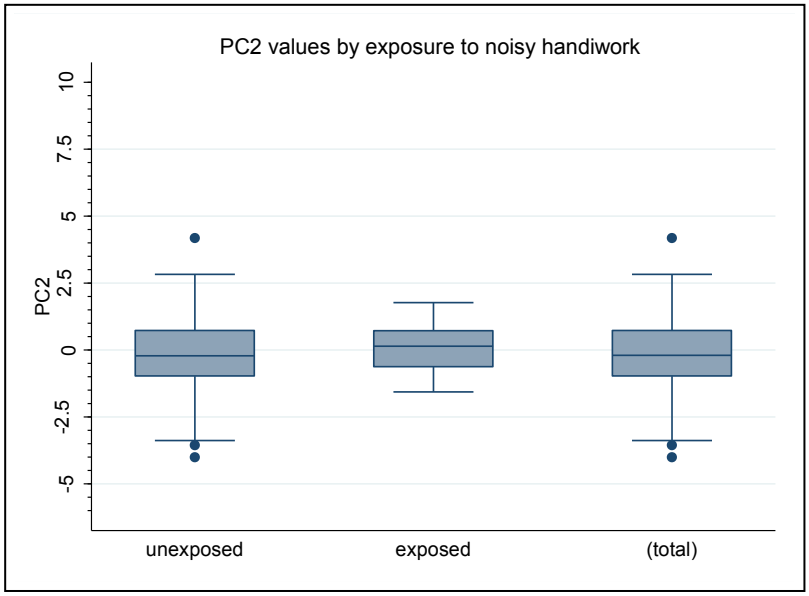




9. Exposure to noisy handiwork

	age-adjusted PC1 (mean±SD)	age-adjusted PC2 (mean±SD)	n
unexposed	-0.0169 ±1.8655	-0.1604 ±1.3297	352
exposed	-0.3503 ±1.7401	0.0619 ±0.9924	12
total	-0.0279 ±1.8602	-0.1531 ±1.3195	364
Student's t-test	p=0.5274	p=0.4652	-





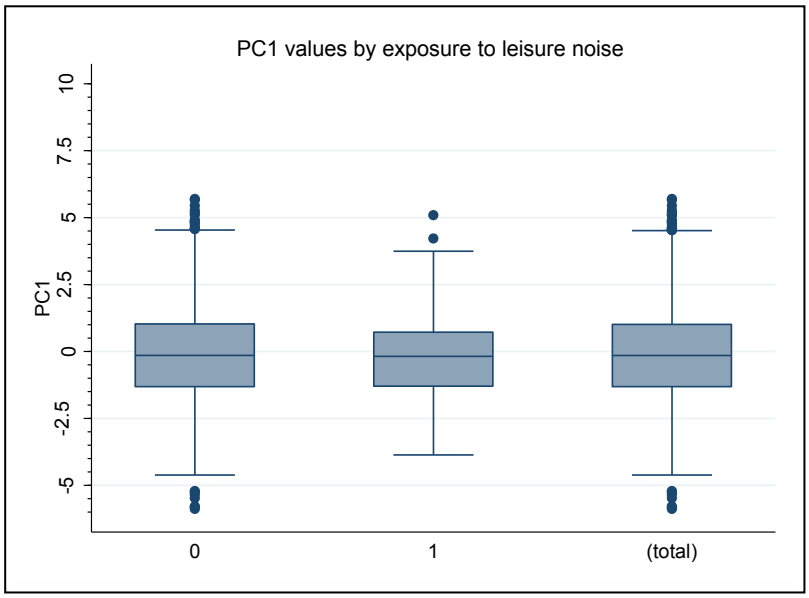
10. Exposure to gunshots

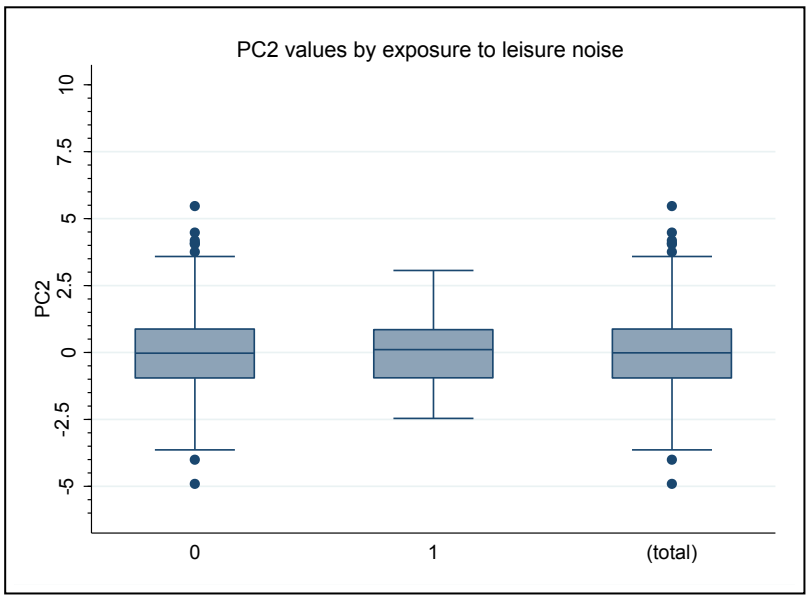
	age-adjusted PC1 (mean±SD)	age-adjusted PC2 (mean±SD)	n
unexposed	-0.0132 ±1.8534	-0.1548 ±1.3229	355
exposed	0.7209	1.7673	1
total	-0.0112 ±1.8512	-0.1494 ±1.3249	356

11. Exposure to noisy leisure activities

(loud music, noisy handiwork and gunshots combined)

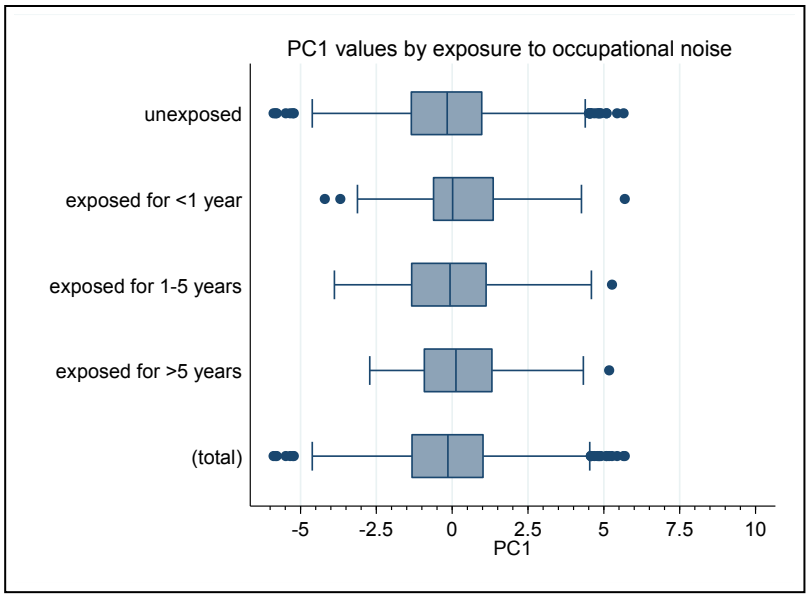
	age-adjusted PC1 (mean±SD)	age-adjusted PC2 (mean±SD)	n
unexposed	-0.0582 ±1.8912	0.0012 ±1.3630	1197
exposed	-0.1222 ±1.7685	0.0653 ±1.3133	98
total	-0.0630 ±1.8816	0.0060 ±1.3589	1295
Student's t-test	p=0.7322	p=0.6440	-

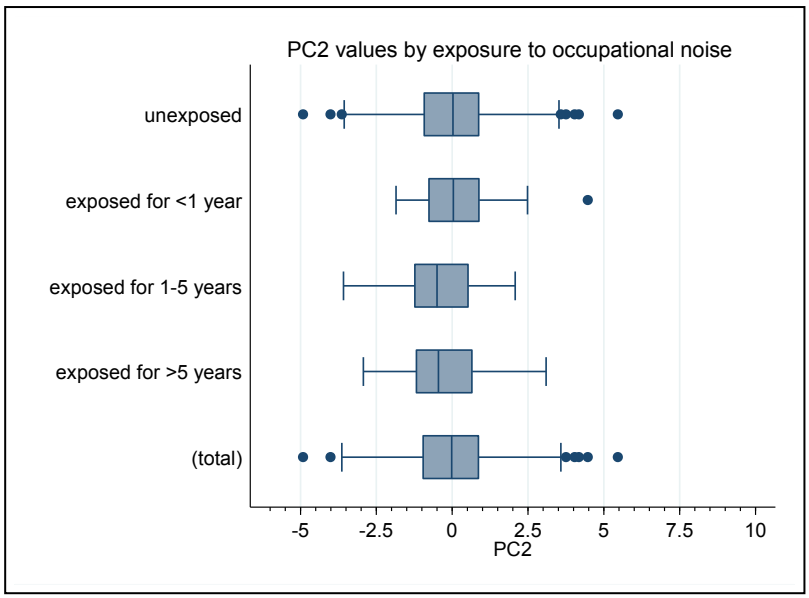




12. Exposure to occupational noise

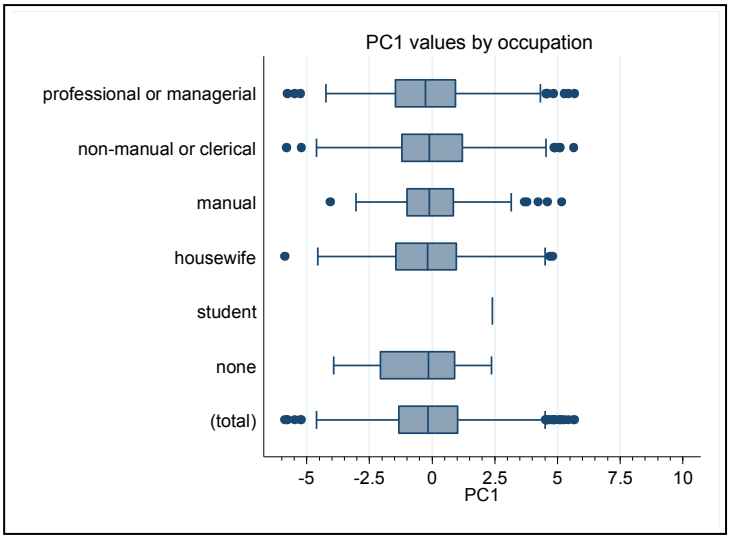
	age-adjusted PC1 (mean±SD)	age-adjusted PC2 (mean±SD)	n
unexposed	-0.0930 ±1.8736	0.0189 ±1.3526	1146
exposed for less than 1 year	0.2967 ±2.0301	0.1402 ±1.2385	50
exposed for 1-5 years	0.1137 ±2.0429	-0.3605 ±1.3061	50
exposed to longer than 5 years	0.2914 ±1.7271	-0.1490 ±1.4880	53
total	-0.0543 ±1.8818	0.0021 ±1.3534	1299
ANOVA	p=0.2205	p=0.1757	-

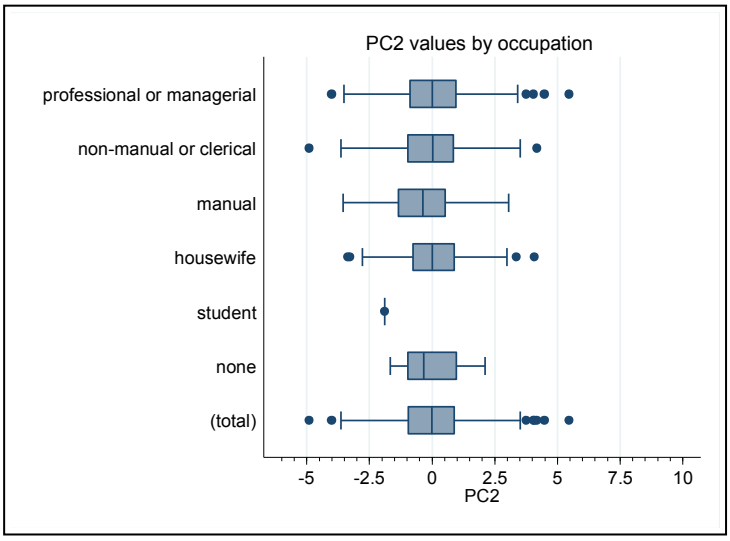




13. Occupation

occupation	age-adjusted PC1 (mean±SD)	age-adjusted PC2 (mean±SD)	n
professional or managerial	-0.2074 ±1.8878	0.0812 ±1.3781	478
non-manual or clerical	0.0857 ±1.8720	-0.0119 ±1.3748	480
manual	0.0463 ±1.7593	-0.3399 ±1.3743	103
housewife	-0.1304 ±1.9205	0.0613 ±1.2484	206
student	2.3992	-1.8943	1
none	-0.4163 ±1.8809	-0.0932 ±1.1546	16
total	-0.0657 ±1.8799	0.0057 ±1.3571	1284
ANOVA	p=0.1209	p=0.0589	-





Appendix chapter 4

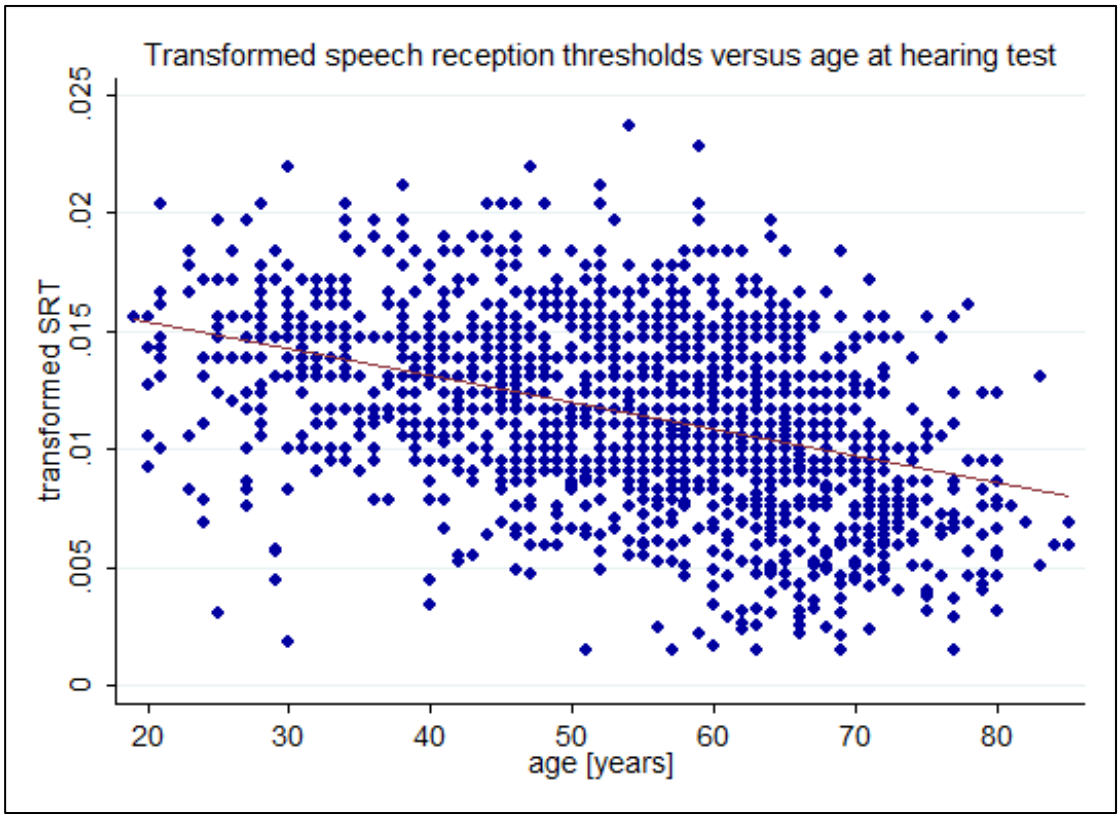


Figure 47 Correlation of age at web-based hearing test with transformed SRTs

This figure shows a scatter plot of chronological age at web-based speech-in-noise hearing test (in years) against transformed speech reception thresholds (SRTs). Chronological age at the test date explained 16.75% of the variance in transformed SRTs. Transformed SRTS decreased with increasing (coef=-0.000011 ± 6.34×10^{-6} se, p=0.000). A linear regression line (red line) was fitted to the dataset.

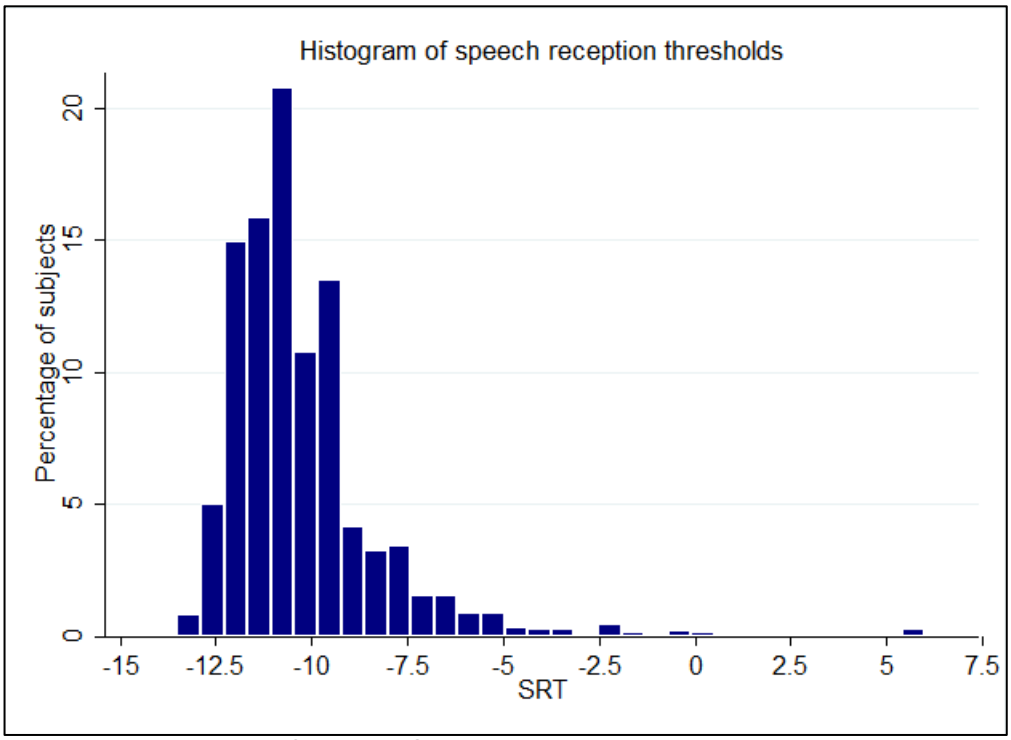


Figure 48 Histogram of original SRTs

The histogram shows the distribution of SRTs in the sample as percentage of subjects from the complete sample with corresponding SRTs.

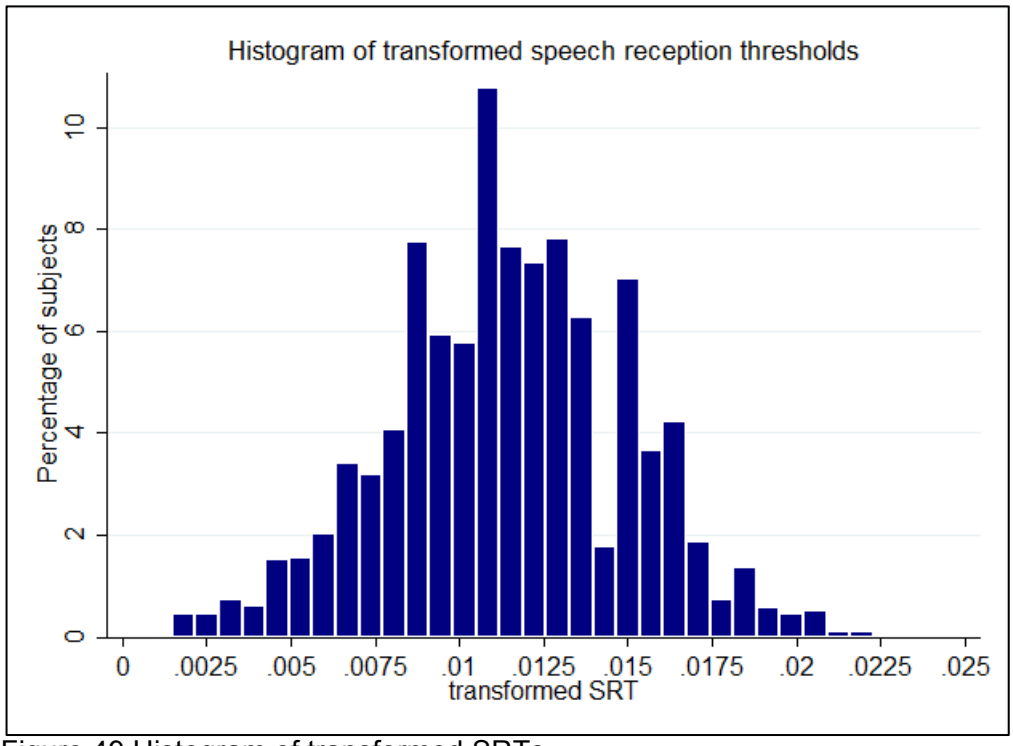


Figure 49 Histogram of transformed SRTs

The histogram shows the distribution of transformed SRTs in the sample as percentage of subjects from the complete sample with corresponding transformed SRTs

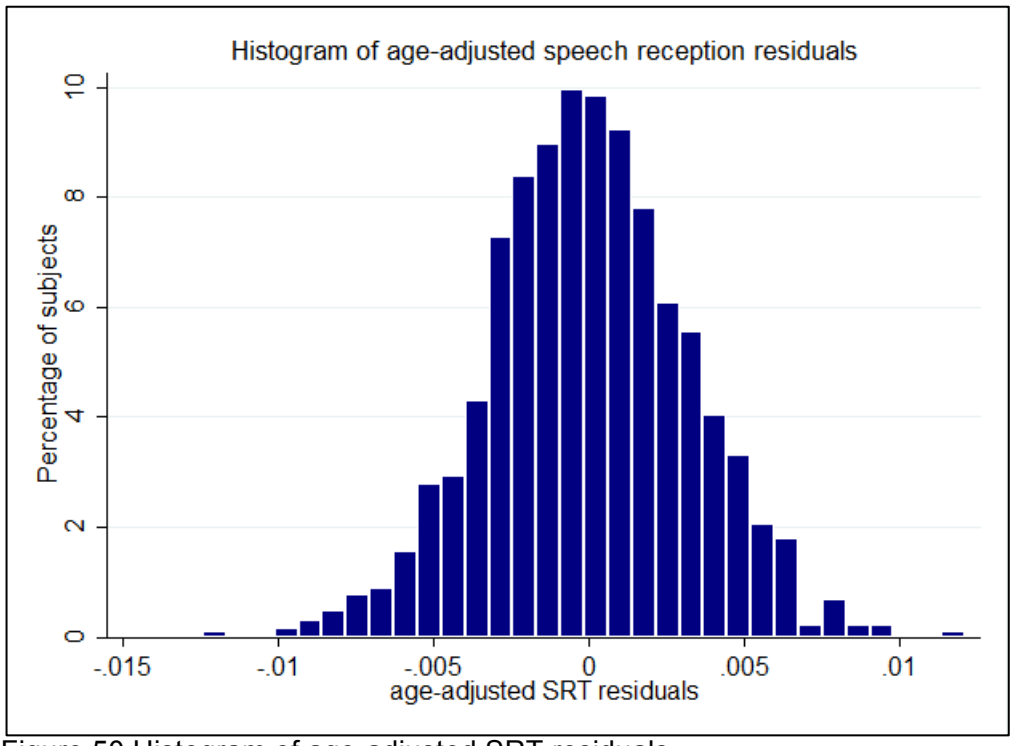


Figure 50 Histogram of age-adjusted SRT residuals

The histogram shows the distribution of age-adjusted SRT residuals in the sample as percentage of subjects from the complete sample with corresponding age-adjusted SRT residuals.

Appendix chapter 5

Table 43 Results of the gene-based association studies

phenotype	chr	gene	nSNPs	gene start gene stop [bp]	test p	p (replication)
SRT	2	FAM110C	5	31607 36385	65.2441266772 3.80E-05	0.2910
	1	COL8A2	20	36333432 36338437	201.3110873808 1.24E-04	0.0966
	1	ZMYM4	6	35507154 35660132	79.0004544011 1.24E-04	0.8560
	1	TEKT2	18	36322262 36326463	171.5698272259 2.42E-04	0.0782
	1	TRAPPC3	21	36374759 36387654	176.2873038451 2.43E-04	0.1190
	1	ADPRHL2	18	36327072 36332120	171.5698272259 2.51E-04	0.0771
	1	KIAA0319L	4	35671677 35795591	34.3522913469 2.87E-04	0.2780
	1	MAP7D1	19	36394389 36419028	157.9870006899 2.92E-04	0.1740
	17	SLFN11	28	30701440 30724833	159.0352029007 3.82E-04	0.3940
	20	LOC400831	47	1132097 1136918	215.5803736878 3.91E-04	0.1670
	10	PLEKHA1	136	124124209 124181856	621.3856505814 4.02E-04	0.6880
	10	ARMS2	72	124204168 124206858	332.5870657010 5.83E-04	0.4590
	3	MME	58	156280129 156384212	267.2805620329 5.92E-04	0.1790
	1	EIF2C3	26	36169358 36294650	237.0368090903 6.10E-04	0.4270
	1	PSMB2	2	35841325 35879730	19.7681755964 6.78E-04	0.5750
	7	NT5C3	77	33020266 33068934	624.0873663299 6.85E-04	0.8400
	7	FKBP9	65	32963576 33013067	457.0516966390 7.26E-04	0.8540
	1	SFPQ	6	35421787 35431322	61.8434006167 7.36E-04	0.7540
	6	ALDH8A1	55	135280220 135312937	353.1985115149 8.00E-04	0.5190
	1	EIF2C4	12	36046414 36093775	108.2195489391 8.66E-04	0.7500
	12	LYZ	53	68028400 68034280	303.4161121183 8.67E-04	0.8790
	1	CLSPN	19	35974404 36008138	177.3196893056 9.52E-04	0.7860
PC1	10	GLRX3	100	131824652 131867860	632.3079471598 2.30E-05	0.4470

phenotype	chr	gene	nSNPs	gene start gene stop [bp]	test p	p (replication)
PC1	6	HIVEP2	187	143114296 143308031	691.7842524914 3.21E-04	0.3730
	3	XIRP1	46	39199710 39209081	218.8959858500 3.81E-04	0.6260
	1	CD53	96	111215343 111244081	546.6698083396 4.00E-04	0.2360
	15	MYO1E	147	57215854 57452363	539.5822646702 4.12E-04	0.1360
	22	XKR3	78	15644305 15682584	392.6700908715 4.51E-04	0.1990
	1	ZNF281	68	198642042 198645789	468.1096139110 5.65E-04	0.8820
	11	**PANX1**	**84**	**93501741** **93554785**	**564.8584080233** **5.65E-04**	**0.0159**
	11	LYVE1	62	10535988 10546941	377.7540548833 6.30E-04	0.5320
	11	TBRG1	24	123997951 124011032	101.6066229251 7.86E-04	0.0507
	5	CHD1	71	98218807 98290138	493.3263905315 8.17E-04	0.8530
	11	RNF141	70	10489800 10519350	374.7735314154 8.19E-04	0.5120
	19	CEBPA	22	38482775 38485160	89.5000362635 9.44E-04	0.8630
PC2	17	DHRS7C	32	9615479 9635326	154.502608706559000 5.50E-05	0.2780
	1	**LYST**	**64**	**233890968** **234096843**	**512.715873463855000** **2.58E-04**	**0.0419**
	17	ARSG	45	63766917 63928595	162.068253832286000 4.71E-04	0.7100
	1	EFNA1	29	153366972 153374010	236.380407010318000 5.00E-04	0.6380
	1	CHRM3	167	237858995 238139340	666.801828593204000 6.30E-04	0.1130
	1	EFNA3	32	153317971 153326638	190.578535943440000 6.44E-04	0.4510
	1	RAG1AP1	27	153374911 153377958	213.738941966987000 7.14E-04	0.5650
	1	DPM3	27	153378990 153379620	209.153402546024000 7.24E-04	0.5880
	1	ADAM15	30	153290385 153301876	163.612411182511000 7.31E-04	0.3840
	1	MUC1	16	153424923 153429324	114.704643616058000 7.31E-04	0.3790
	1	TRIM46	17	153412983 153424069	124.834160464716000 7.46E-04	0.3740
	6	BMP6	156	7672009 7826960	570.522527358666000 8.32E-04	0.6320
	11	LOC644672	58	113155727	276.627931290039000	0.7160

phenotype	chr	gene	nSNPs	gene start gene stop [bp]	test p	p (replication)
				113156417	8.37E-04	
	1	EFNA4	33	153302836 153308653	174.074028976508000 8.58E-04	0.4680
	1	THBS3	15	153432002 153444314	103.903100592252000 9.11E-04	0.3580
unadjusted PTA	10	GLRX3	100	131824652 131867860	662.7044512171 1.00E-05	-
	13	GPR12	52	26230959 26231964	325.8311958210 1.28E-04	-
unadjusted PTA	5	CHD1	71	98218807 98290138	590.8798504793 2.13E-04	-
	3	CHCHD6	139	127905807 128161934	822.2741781330 3.21E-04	-
	19	ELSPBP1	55	53189742 53220217	244.2502769688 3.89E-04	-
	19	CABP5	55	53225021 53239116	238.6667832603 4.18E-04	-
	5	REEP5	62	112239979 112285930	532.3056059406 4.32E-04	-
	6	HEY2	29	126112424 126124108	231.5221231219 4.38E-04	-
	19	BSPH1	46	53163114 53187239	217.1527189269 4.53E-04	-
	7	WNT16	34	120752656 120768394	227.5994727882 5.16E-04	-
	17	FN3KRP	33	78267894 78279146	202.7587557149 5.50E-04	-
	1	TMCO4	63	19881292 19998997	227.7438824274 5.78E-04	-
	17	FN3K	36	78286796 78302362	255.6953860606 6.17E-04	-
	5	SRP19	52	112224891 112231503	496.2085120380 6.22E-04	-
	8	TAF2	175	120812194 120914255	927.4767480306 6.87E-04	-
	1	HTR6	42	19864366 19878642	188.4830896844 8.38E-04	-
	1	SDCCAG8	97	241485942 241730016	462.6429356037 9.51E-04	-
	1	NBL1	43	19842312 19857532	190.9223714250 9.68E-04	-
age-adjusted PTA	10	GLRX3	100	131824652 131867860	542.4649639709 1.14E-04	0.4470
	15	MYO1E	147	57215854 57452363	596.3068102455 1.73E-04	0.1360
age-adjusted PTA	**7**	**WNT16**	**34**	**120752656 120768394**	**245.0590637250 3.35E-04**	**0.0107**
	3	STAC	172	36397100 36564500	701.3925443186 3.98E-04	0.4470

phenotype	chr	gene	nSNPs	gene start gene stop [bp]	test p	p (replication)
	3	XIRP1	46	39199710 39209081	200.5656241959 8.74E-04	0.6260
	19	TDRD12	64	37902518 37973554	480.6724799050 8.74E-04	0.1380
	6	ZNF318	43	43411785 43445159	280.0812249136 9.77E-04	0.3620

This table presents the results of the gene-based association studies for different hearing function and ARHI phenotypes, respectively. Phenotypes included age and gender-adjusted SRT residuals (SRT) and age-adjusted PC1 (PC1), PC2 (PC2) and PTA (PTA) residuals. The results of the gene-based analysis using VEGAS are presented by gene, chromosomal location of the respective gene (chr, gene start and gene stop), number of SNPs tested per gene (nSNPs), the gene-based test statistic (test) and the level of significance of association (p). For each gene 10^6 simulations were performed. The p-value achieved in the gene-based replication study is listed as p (replication). Significantly replicated associations are presented in bold.

Appendix chapter 6

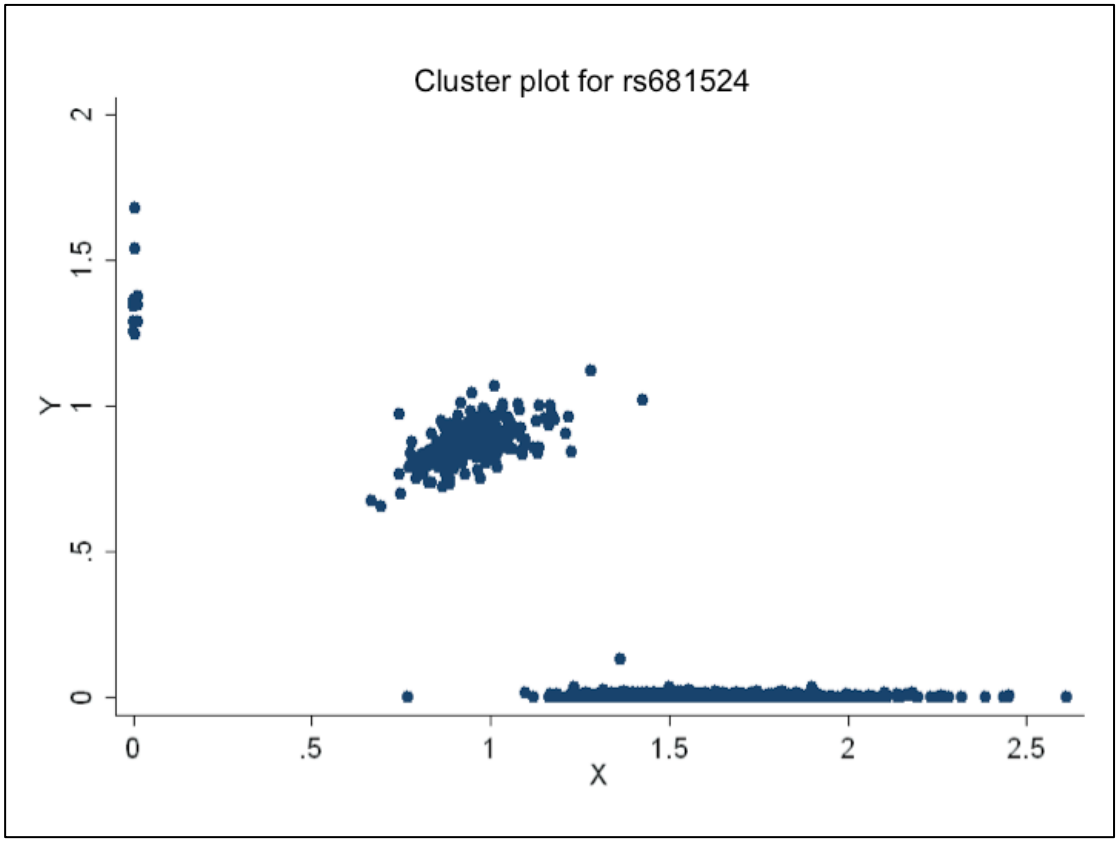


Figure 51 Genotyping cluster plot for rs681524 in TwinsUK

The genotyping cluster plot shows clear separation of genotyping signals for the two alleles. Signal on the y-axis represents the genotyping signal for the C-allele, while the x-axis represents the genotyping signal for the T-allele.

Table 44 Genotyping and imputation information per population

Population	array	genotype calling	QC criteria	imputation	imputation reference population	statistical analysis tool
Carlantino	Illumina 370 CNV	Bead studio	call rate>90% p(hwe)>10^{-4}	MACH	HapMap Phase II	GenABEL, ProbABEL
Friuli Venezia Guilia	Illumina 370 CNV	Bead studio	call rate>90% p(hwe)>10^{-4}	MACH	HapMap Phase II	GenABEL, ProbABEL
Korcula	Illumina 370 CNV	GenomeStudio	call rate>98% p(hwe)>10^{-10}	MACH	HapMap Phase II	GenABEL, ProbABEL
Split	Illumina 370 CNV	GenomeStudio	call rate>98% p(hwe)>10^{-10}	MACH	HapMap Phase II	GenABEL, ProbABEL
Cilento	Illumina 370 K	Illumina	call rate>95% SNPs not in HapMap	MACH	HapMap Phase II	GenABEL, ProbABEL
Talana	Affymetrix 500 K	BRLMM	p(hwe)<10^{-6} call rate 95%, MAF≥1%	MACH	HapMap Phase II	GenABEL, ProbABEL
Silk Road	Illumina 700 K	GenomeStudio	call rate>97% p(hwe)>10^{-8}	MACH	HapMap Phase II	GenABEL, ProbABEL
TwinsUK	Illumina HumanHap300 Bead Chip Illumina HumanHap610 Quad Chip	Illuminus algorithm	call rate≥97% (SNPs with MAF≥5%) call rate≥99% (for SNPs with 1%≤MAF<5%) p(hwe)>10^{-6} MAF≥1%	Impute vs2	HapMap Phase II	GenABEL

The number of genotyped SNPs taken forward for imputation was based on passing the defined quality control (QC) criteria (p(hwe)= significance of deviation from Hardy Weinberg equilibrium, MAF=minor allele frequency, call rate=genotyping efficiency per SNP). Imputation was performed using Markov Chain based haplotyper (MACH) or Impute vs2 based on the HapMap Phase II CEU reference population. As statistical tools for genome-wide association analyses ProbABLE and GenABEL in R were used.

Table 45 Meta-analysis results for PC1

SNP	allele1 allele2	Z-score	p-value	direction	chr	position	gene	feature	left gene right gene
rs589636	t c	-5.40	6.61E-08	?------?	13	76414577	*IRG1*	intron	*BTF3L1* *LOC390413*
rs588702	t c	-5.33	9.75E-08	?-------	13	76420926	*IRG1*	intron	*BTF3L1* *LOC390413*
rs2687481	t g	-5.32	1.07E-07	-------?	7	125656358	NA	NA	*LOC646837* *GRM8*
rs2521030	c g	-5.23	1.69E-07	-------?	7	125656552	NA	NA	*LOC646837* *GRM8*
rs614171	a g	-5.23	1.71E-07	?-------	13	76414753	*IRG1*	intron	*BTF3L1* *LOC390413*
rs592425	a g	4.96	7.21E-07	?++++?++	13	76438632	*IRG1*	intron	*BTF3L1* *LOC390413*

Six single nucleotide polymorphisms (SNPs) were suggestive genome-wide significant ($p<0.5x10^{-7}$) associated with PC1. Meta-analysis results for PC1 are further characterised by non-effect allele (allele1) and effect allele (allele2), the resulting Z-score and significance of association (p-value). The direction of effect (minus or plus) is indicated for each of the 8 included populations. If a SNP did not pass QC criteria for a certain SNP, this is indicated by a question mark (?) in the direction column. Mapping information for each SNP is specified by chromosome (chr), base-pair position (position), genes at this locus (gene) and surrounding the locus (left and right gene) as well as feature of the SNP position within a gene (feature).

Table 46 Meta-analysis results for PC2

SNP	allele1 allele2	Z-score	p-value	direction	chr	position	gene	feature	left gene right gene
rs681524	t c	-5.505	3.69E-08	?-------	11	116253524	*SIK3*	intron	*APOA1* *LOC100129905*
rs1393902	a g	5.119	3.07E-07	++++++++	8	68584119	*CPA6*	intron	*ARFGEF1* *LOC100132812*
rs1503369	t c	5.116	3.12E-07	++++++++	8	68584552	*CPA6*	intron	*ARFGEF1* *LOC100132812*
rs1827524	a g	5.104	3.32E-07	++++++-+	8	68587796	*CPA6*	intron	*ARFGEF1* *LOC100132812*
rs1393901	t c	5.100	3.40E-07	++++++-+	8	68587888	*CPA6*	intron	*ARFGEF1* *LOC100132812*
rs6472312	t g	-4.994	5.92E-07	------+-	8	68572052	*CPA6*	intron	*ARFGEF1* *LOC100132812*
rs1503363	a g	-4.940	7.80E-07	------+-	8	68569258	*CPA6*	intron	*ARFGEF1* *LOC100132812*

Seven single nucleotide polymorphisms (SNPs) were suggestive genome-wide significantly ($p<0.5x10^{-7}$) associated with PC2. Top associated SNPs for PC2 are further characterised by corresponding non-effect allele (allele1) and effect allele (allele2), the resulting Z-score and significance of association (p-value). The direction of effect (minus or plus) is indicated for each of the 8 included populations. If a SNP did not pass QC criteria for a certain SNP, this is indicated by a question mark (?) in the direction column. Mapping information for each SNP is specified by chromosome (chr), base-pair position (position), genes at this locus (gene) and surrounding the locus (left and right gene) as well as feature of the SNP position within a gene (feature).

Appendix chapter 7

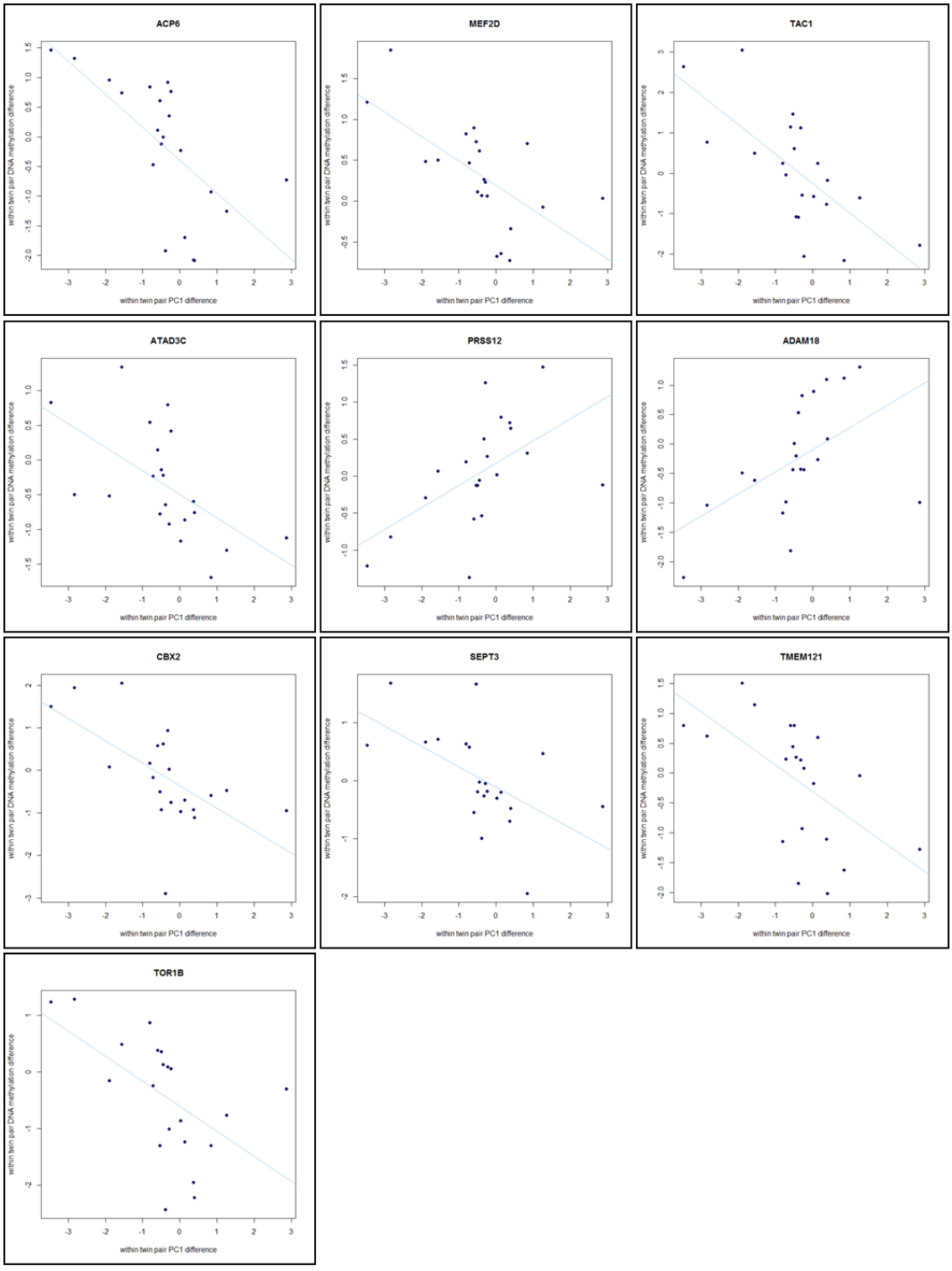


Figure 52 Differentially methylated regions in MZ twin pairs discordant for PC1

Scatter plots are shown for the ten probes at which differential DNA methylation within twin pairs was most strongly correlated with within pair differences in hearing PC1 (Table 4). The difference in DNA methylation within twin pairs was calculated as within pair difference in DNA methylation residuals (adjusted for batch effects). A linear regression line (lightblue line) was fitted to each scatter plot.

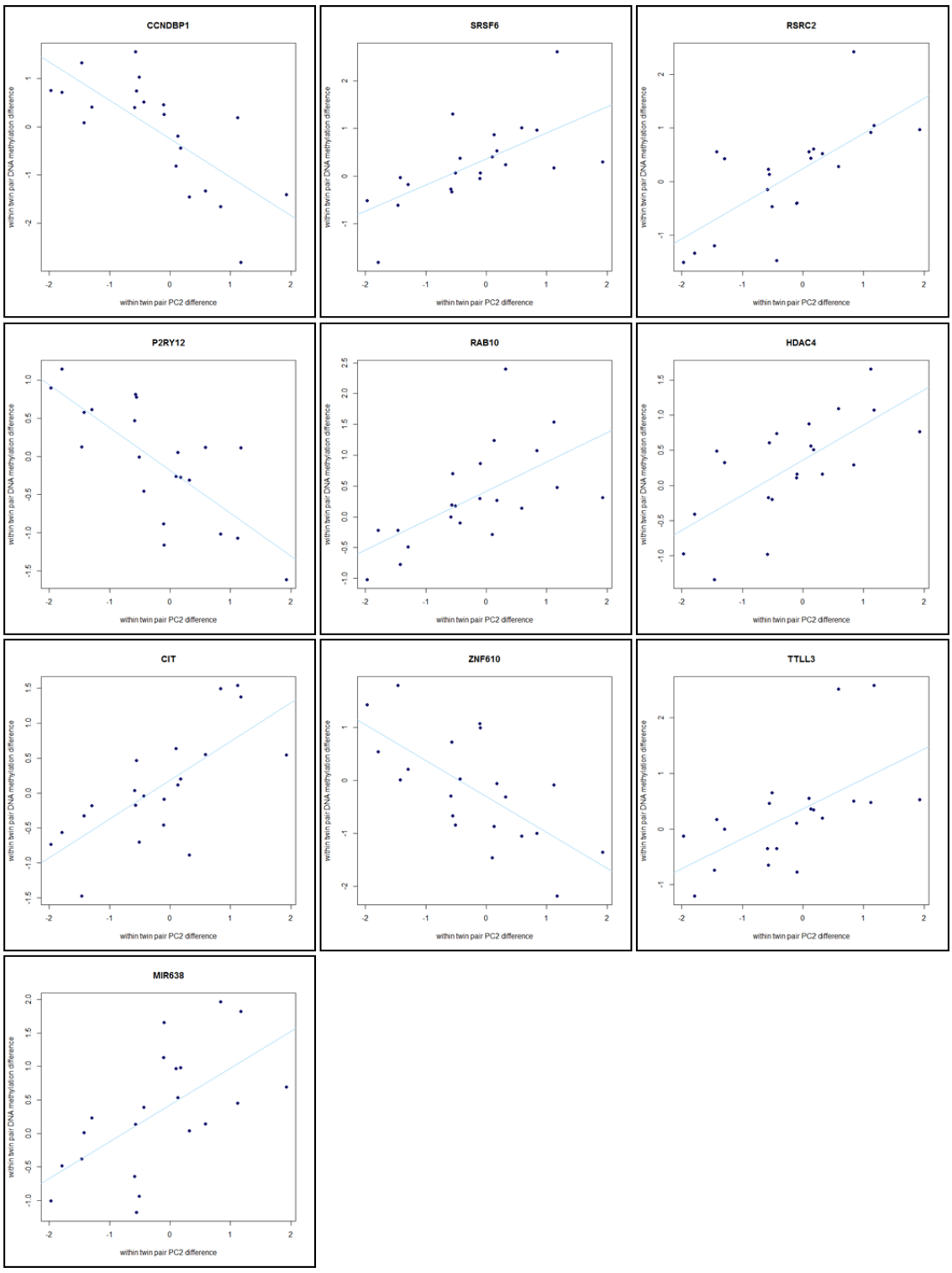


Figure 53 Differentially methylated regions in MZ twin pairs discordant for PC2

Scatter plots are shown for the ten probes at which differential DNA methylation within twin pairs was most strongly correlated with within pair differences in hearing PC2 (Table 4). The difference in DNA methylation within twin pairs was calculated as within pair difference in DNA methylation residuals (adjusted for batch effects). A linear regression line (lightblue line) was fitted to each scatter plot.